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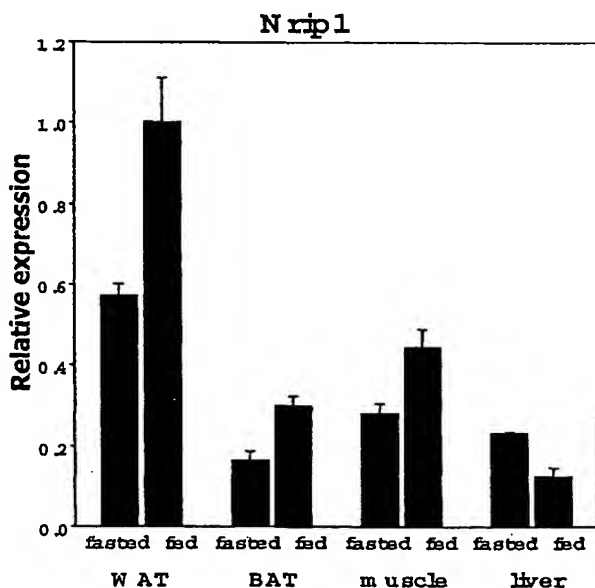
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(54) Title: SCREENING FOR MODULATORS OF FAT STORAGE



(57) Abstract: This invention relates to screening methods for identifying compounds that are useful as modulators of fat storage. In particular, the invention provides methods of screening for compounds that modulate the function of receptor interacting protein 140 (RIP 140).

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SCREENING FOR MODULATORS OF FAT STORAGE

All documents cited herein are incorporated by reference in their entirety

TECHNICAL FIELD

This invention relates to screening methods for identifying compounds that are useful as
5 modulators of fat storage. In particular, the invention provides methods of screening for
compounds that modulate the function of receptor interacting protein 140 (RIP140).

BACKGROUND ART

Conditions which involve a significant increase in fat storage, such as obesity, and conditions
which involve a significant decrease in fat storage, such as anorexia, are associated with severe
10 health problems. Obesity is associated with diseases such as diabetes, heart disease and
hypertension while anorexia can cause irreversible bone damage and ultimately death.

In the past, such conditions have been primarily identified as having psychological causes.
However, there is increasing recognition that genetic mechanisms are also involved in conditions
where there is a significant deviation in fat storage (Schalling *et al*, J Intern Med 1999 Jun;
15 245(6): 613-9; Spiegelman & Flier, Cell 2001 Feb; 104: 531-543). For example, the *ob* gene has
been suggested to be essential to the control of fat storage and it has been suggested that the
leptin polypeptide encoded by the *ob* gene could be used to treat obesity. However, transgenic
mouse models used to test the effect of different genes associated with the modulation of fat
storage have not always had the expected phenotype (Arch, J Endocrinol Invest 2002 Nov;
20 25(10):867-75). There is therefore a need to identify further compounds that modulate fat
storage. Thus, an object of the invention is the provision of a method for identifying compounds
that modulate fat storage.

DISCLOSURE OF THE INVENTION

The nuclear receptor interacting protein 140 (RIP140), also known as Nrip1 (nuclear receptor
25 interacting protein 1), is a co-activator or co-repressor of transcription by a variety of nuclear
receptor family members including the estrogen receptors (ER), such as estrogen receptor α
(ER α) and estrogen related receptor α (ERR α), retinoic acid receptors (RAR), thyroid hormone
receptors (TR), retinoid X receptors (RXR), vitamin D receptor (VDR) and peroxisome
proliferator activated receptors (PPAR α , PPAR δ (also known as PPAR β) and PPAR γ), as well as
30 by the aryl hydrocarbon receptor (AhR). It has been also been found to modulate transcription of
the steroidogenic acute regulatory protein gene (StAR) through interactions with the

transcription factors steroidogenic factor 1 (SF-1; also known as Ad4BP) and DAX-1 (Sugawara *et al*, 2001, *Endocrinology* 142: 3570-3577).

RIP140 is known to be essential for female fertility. Female mice null for RIP140 are viable but infertile because of complete failure to release oocytes at ovulation (White *et al*, 2000, *Nature Medicine*, 6:1368-1374). RIP140 is thought to have a secondary role in maintenance of pregnancy (Leonardsson *et al*, 2002, *Endocrinology*, 143(2): 700-707).

Surprisingly, it has now been discovered that RIP140 plays a role in controlling fat storage. Mice devoid of the corepressor protein RIP140 are leaner than wild-type mice and fat in food-deprived RIP140 null mice appears to be deposited in muscle cells in greater amounts than in food-deprived wild-type mice. RIP140 null mice are resistant to high-fat diet induced obesity and hepatic steatosis. Fat accumulation in white adipose tissue (WAT) is decreased in RIP140 null mice compared to wild-type mice.

In addition, genes that are expressed only in brown adipose tissue (BAT) in wild-type mice are to be expressed in the WAT of mice null for RIP140. Unlike WAT, BAT can be used as an energy source as a result of uncoupling. Genes involved in fat metabolism, energy dissipation and mitochondrial uncoupling are markedly induced in the WAT of RIP140 null mice, resulting in an increase in oxygen consumption. One of the genes that is expressed at higher levels in the WAT of RIP140 null mice is uncoupling protein 1 (UCP1), a key regulatory gene involved in fat metabolism that is typically expressed in the BAT and plays a major role in uncoupling ATP synthesis from respiration (Lowell, B.B. & Spiegelman, B.M., 2000, *Nature* 404: 652-60; Kozak, L.P. & Harper, M.E., 2000, *Annu Rev Nutr* 20: 339-63).

It is believed that the RIP140 corepressor prevents mitochondrial uncoupling in the WAT of wild-type mice and the leanness of mice null for RIP140 results from an ability to use WAT as an energy source. Adipogenesis appears to be unaffected in RIP140 null mice but it is also possible that the leanness of RIP140 null mice results from RIP140 having a role in adipocyte function which is inhibited in the null mice.

Because RIP140 null mice have decreased fat storage, compounds that modulate the activity of RIP140 may modulate fat storage. RIP140 is a co-regulator which exerts its effects by binding to nuclear receptor family members such as ER, RAR, TR, RXR, VDR or PPAR or transcription factors such as AhR, SF-1 and DAX-1. These proteins to which RIP140 specifically binds are referred to herein as target proteins. Compounds may modulate the activity of RIP140 by promoting or inhibiting its ability to form a complex with one or more of these target proteins.

Compounds that modulate the ability of RIP140 to form a complex with one or more of these target proteins may act to decrease or increase fat storage. Compounds that bind to RIP140 may promote or inhibit its ability to form a complex with a target protein. Similarly, compounds that bind to a target protein of RIP140 may promote or inhibit the ability of RIP140 to form a complex with that target protein.

RIP140 is shown herein to target a regulatory region in the promoter of the UCP-1 gene which is one of the genes upregulated in RIP140 null mice. This regulatory region contains binding sites for PPAR, TR, RXR and RAR, suggesting that RIP140 may act as a co-repressor of one or more of these target proteins. Compounds that modulate the ability of RIP140 to form a complex with one or more of PPAR, TR, RXR and RAR may act to decrease or increase fat storage. Compounds that bind to RIP140 may promote or inhibit its ability to form a complex with PPAR, TR, RXR and RAR. Similarly, compounds that bind to PPAR, TR, RXR and RAR may promote or inhibit the ability of PPAR, TR, RXR and RAR to form a complex with RIP140. As a first method, the invention therefore provides a method of screening for compounds that bind to RIP140, said method comprising assessing binding of a candidate compound to RIP140. The invention further provides a method of screening for compounds that bind to a target protein of RIP140, said method comprising assessing binding of a candidate compound to the target protein. The target protein of RIP140 is preferably selected from AhR, ER, RAR, TR, RXR, VDR, PPAR, SF-1 and DAX-1. Preferably, the target protein of RIP140 is selected from PPAR, TR, RXR and RAR.

Preferably, the target protein of RIP140 is selected from PPAR α , PPAR δ and PPAR γ . These three nuclear receptors have already been shown to have a role in fat metabolism. Previous work has shown that PPAR γ plays an essential role in adipogenesis (Lazar, M.A, 2002, *Genes Dev*, 16: 1-5; Mueller, E. *et al.*, 2002, *J Biol Chem* 277: 41925-30; Ren, D. *et al.*, 2002, *Genes Dev*, 16: 27-32 (2002); Barak, Y. *et al.*, 1999, *Mol Cell* 4: 585-95). PPAR α has been shown to be involved in thermogenesis and fatty acid oxidation (Kelly, D.P, 2003, *Circ Res*, 92: 482-4; Peters, J.M. *et al.*, 1997, *J Biol Chem*, 272: 27307-12; Barbera, M.J. *et al.*, 2001, *J Biol Chem*, 276: 1486-93) and PPAR δ has been implicated in lipid homeostasis (Wang, Y.X. *et al.*, 2003, *Cell* 113: 159-70).

As a second method, the invention provides a method of screening for compounds that bind to a complex of RIP140 and a target protein, said method comprising assessing binding of a candidate compound to a complex of RIP140 and a target protein.

The invention further provides a third method of screening for compounds that modulate the formation or maintenance of a complex of RIP140 and a target protein. A modulator may be either an agonist or an antagonist of the RIP140:target protein interaction. An agonist is a compound that promotes the formation and/or maintenance of a complex of RIP140 and a target protein. An antagonist is a compound that inhibits the formation and/or maintenance of a complex of RIP140 and a target protein.

Preferably, the method of screening for compounds that act to modulate the formation or maintenance of a complex of RIP140 and a target protein comprises the steps of:

(a) mixing RIP140, a target protein and one or more candidate compounds;

(b) incubating the mixture to allow RIP140, the target protein and the candidate compound(s) to interact; and

(c) assessing whether the interaction between RIP140 and the target protein is modulated.

The mixing of RIP140, the target protein and candidate compound in step (a) may be done in any order.

The candidate compounds used in the third method may be compounds already identified as binding to RIP140 or a target protein by the first method of the invention, or compounds identified as binding to a complex of RIP140 and a target protein by the second method of the invention.

***In vivo* confirmation of function of compounds identified**

Once a candidate compound has been identified *in vitro* as a compound that binds to RIP140 or to a target protein, as a compound that binds to a complex of RIP140 and a target protein, or as a modulator of the interaction between a target protein and RIP140, it may be desirable to perform further experiments to confirm the *in vivo* function of the compound in modulating fat storage. Any of the above methods may therefore comprise the further steps of administering to a mammal a candidate compound and assessing its effect on fat storage.

The invention also provides a method of assessing the *in vivo* effect on fat storage of a compound obtained or obtainable by any of the methods described above, comprising administering the compound to a mammal and assessing the effect on fat storage.

In general, therefore, the invention provides a method for screening for a compound that modulate fat storage in a mammal, comprising a first step of identifying a compound which: (a) binds to RIP140 or to a RIP140 target protein; (b) binds to a complex of RIP140 and a

RIP140 target protein; or (c) modulates the binding interaction between RIP140 and a RIP140 target protein, and comprising a second step of administering to a mammal a candidate compound identified in step (a), (b) or (c) and assessing its effect on fat storage in the mammal.

The mammal may be any species of mammal including humans but is preferably a monkey, pig, rabbit, guinea pig, rat or mouse. Tests on non-humans may be preferred.

The compound identified by a method of the invention may be administered to animal models of obesity and leanness. The compound may be administered to an animal in which a gene or combination of genes known to be involved in fat storage has been knocked-out. For example, the compound may be administered to an animal that is null for RIP140 or an animal that is null for the *ob* gene. Other models are widely available (*e.g.* Pomp (1999) *Molecular Medicine Today* 5:459-460).

The methods of screening for compounds that bind to RIP140 or to a target protein, or that bind to a complex of RIP140 and a target protein, or that modulate the interaction between RIP140 and a target protein may take place in a different geographical location from the method for assessing the effect of the compounds identified on fat storage.

Direct screening for compounds that bind to RIP140 or to a target protein, or that bind to a complex of RIP140 and a target protein

The RIP140 or target protein used in the method of screening for compounds that bind RIP140 or a target protein may be free in solution, affixed to a solid support, located on a cell surface or located intracellularly.

Preferably, the binding of a candidate compound to RIP140 or to a target protein is detected by means of a label directly or indirectly associated with the candidate compound. The label may be a fluorophore, radioisotope, or other detectable label.

For example, in a method of screening whether a candidate compound binds to RIP140, one or both of the candidate compound and RIP140 may be labelled with a fluorescent label such that the binding between the candidate compound and RIP140 may be detected by an intrinsic fluorescence change which occurs when the candidate compound binds to RIP140. For example, the candidate compound may be joined to a fluorescence resonance energy transfer (FRET) donor and RIP140 to a FRET acceptor (or *vice versa*) such that, when the candidate compound and RIP140 interact, stimulation of the FRET donor excites the FRET acceptor causing it to emit

photons. Interaction may be also be detected by fluorescent labelling of the candidate compound and/or RIP140 such that fluorescence is quenched when they form a complex.

Other methods for assessing interaction between the candidate compound and RIP140 or between a candidate compound and a target protein may include using NMR to determine whether a RIP140:candidate compound complex or a target protein:candidate compound complex is present.

The presence of a RIP140:candidate compound complex or a target protein:candidate compound complex may also be detected as a band at a particular position when run on a gel.

Another method of assessing interaction between RIP140 and a candidate compound may involve immobilising RIP140 on a solid surface and assaying for the presence of free candidate compound. If there is no interaction between the candidate compound and RIP140, free candidate compound will be detected. The candidate compound may be labelled to facilitate detection. This type of assay may also be carried with the candidate compound being immobilised on the solid surface. Interaction between the immobilised RIP140 and the free candidate compound may also be monitored by a process such as surface plasmon resonance.

Other methods for studying RIP140 interactions are described by Sugawara *et al*, 2001, *Endocrinology* 142: 3570-3577.

These techniques described above for use with RIP140 can, of course, be used *mutatis mutandis* for any of the target proteins of the invention *e.g.* to detect whether a candidate compound binds to a target protein.

The methods of screening for compounds that bind to a complex of RIP140 and a target protein can be carried out using the methods described above but using a complex of RIP140 and a target protein in place of RIP140 or in place of the target protein.

Direct screening for modulators of a complex of RIP140 and a target protein

Modulation of the interaction between RIP140 and a target protein in the presence of candidate compounds may be assessed directly. Various methods for direct detection of protein/protein interactions are available.

The methods described above for assessing whether a candidate compound binds to RIP140, to a target protein, or to a complex of RIP140 and a target protein, can also be used to assess whether a candidate compound modulates the interaction between RIP140 and a target protein. The target protein is already known to bind RIP140 and the methods described above are hence used to

assess whether that interaction is disrupted or promoted by the candidate compound.

For example, one of both the target protein and RIP140 may be labelled with a fluorescent label so that interaction between the target protein and RIP140 may be detected by an intrinsic fluorescence change that occurs when the RIP140:target protein complex is formed or disrupted in the presence of a candidate compound.

Interaction of RIP140 and a target protein in the presence of candidate compound may also be assessed by detecting the accessibility of peptide sequences (*e.g.* epitopes) on RIP140 and/or the target protein that are masked when the two proteins form a complex. For example, motifs on RIP140 that interact with nuclear receptors have been identified in WO98/49561, Lee *et al* (Mol Cell Biol, 1998, 18(11): 6745-44) and in Wei *et al* (J. Biol. Chem., 2001, 276(19): 16107-12). A lack of interaction between RIP140 and a target protein that is a nuclear receptor in the presence of a candidate compound may therefore be determined by detection of such motifs, for example, using antibodies.

Indirect screening for modulators of RIP140:target protein complex formation and maintenance using two-hybrid systems

Indirect methods for assessing whether the interaction between a target protein and RIP140 is modulated in the presence of a candidate compound may also be used. One indirect method of screening for modulation of the interaction between target protein and RIP140 in the presence of a candidate compound involves using a two-hybrid system. The target protein may be fused to an activation domain of a transcription factor and RIP140 to a DNA-binding domain of a transcription factor (or *vice versa*), such that interaction between the target protein and RIP140 promotes the transcription of a reporter gene in a cell.

The invention provides a method of screening for compounds that modulate the interaction between the target protein and RIP140, said method comprising:

- a) contacting a cell containing a nucleic acid molecule comprising a promoter operatively linked to a reporter gene with: (i) a first fusion protein comprising one of the target protein and RIP140 fused to the activation domain of a transcription factor, (ii) a second fusion protein comprising the other of the target protein and RIP140 fused to the DNA-binding domain of a transcription factor; and (iii) a candidate compound; and
- b) assessing the level of expression of the reporter gene,

wherein interaction between the target protein and RIP140 promotes transcription of the reporter gene by activating said promoter.

This method may be used to assess interaction between RIP140 and the target protein in any eukaryotic cell. Preferably, the method is used to assess the interaction between RIP140 and the target protein in a yeast cell or a mammalian cell. Where the candidate compound is an organic compound and a yeast two-hybrid system is being used, the permeability of the yeast cell wall is preferably enhanced *e.g.* by using chemicals such as polymyxin B.

The level of expression of a reporter gene in the two-hybrid system is indicative of the level of interaction between the target protein and RIP140. A candidate compound that inhibits the interaction between the target protein and RIP140 decreases or abolishes the level of expression of the reporter gene. A candidate compound that promotes the interaction between the target protein and RIP140 maintains or increases the level of expression of the reporter gene.

Preferably, the reporter gene is easily assayed. For example, the reporter gene may give a detectable signal, such as a visible signal. The reporter gene may encode a protein which gives a visible signal itself, or which catalyses a reaction which gives a visible signal or change *e.g.* a fluorescent protein or an enzyme. The reporter gene may encode an enzyme such as a beta-galactosidase or a peroxidase, both of which are commonly used with coloured substrates and/or products. The reporter gene may encode a green fluorescent protein (GFP) or a fluorescent derivative thereof such as YFP or CFP (see Prasher *et al.*, 1995, *Trends Genet* 11(8): 320). The reporter gene may encode a luminescent protein, such as luciferase.

The reporter gene may drive DNA replication (Vasavada *et al.*, 1991, *PNAS*, 88:10686-10690) in the cell or may encode a drug resistance marker (Fearon *et al.*, 1992, *PNAS* 89: 7958-7962).

The reporter gene may encode a protein that enables positive selection of cells in which the interaction between the target cell and RIP140 is inhibited. For example, the reporter gene may encode a protein that is toxic or cytostatic so that only cells that do not express the reporter are able to survive or grow. As a result, the only cells to survive are those in which a candidate compound inhibits the interaction between the target protein and RIP140 so that the reporter gene is not expressed. Examples of reporter genes of this type that may be used in yeast include URA3, LYS2 and CYH2 (see Vidal *et al.*, 1996, *PNAS*, 93: 10315-10320). The protein encoded by the reporter gene may also prevent cell growth in the absence or presence of a particular amino acid or other component in cell media. For example, the reporter gene may encode a DNA-binding protein, Tn10 tetracycline, which represses transcription of a TetRop-HIS3 gene

so that yeast cells in which the reporter gene is expressed do not grow in the absence of histidine (see Shih et al, 1996, *PNAS*, 93: 13896-13901). In contrast, yeast cells in which the interaction between RIP140 and the target protein has been disrupted do not express TN10 tetracycline and are consequently able to grow in the absence of histidine.

- 5 The proteins encoded by the reporter genes may be in the form of fusion proteins. Methods for the generation of fusion proteins are standard in the art and will be known to the skilled reader. For example, most general molecular biology, microbiology recombinant DNA technology and immunological techniques can be found in Sambrook *et al.*, (Molecular Cloning, A Laboratory Manual, Cold Harbor-Laboratory Press, Cold Spring Harbor, N.Y., 2000) or Ausubel *et al.*,
10 (Current Protocols in Molecular Biology, Wiley Interscience, NY, 1991).

Other indirect screening methods for modulators of RIP140:target protein interaction

- Many of the target proteins of RIP140 are transcription factors which are activated or inhibited by interaction RIP140. For example, SF-1 is a transcription factor which is inhibited by RIP140 while transcription of genes by AhR is activated by RIP140 (Kumar *et al.*, J. Biol. Chem. (1999)
15 274:22155-22164). Interaction between RIP140 and a target protein of this type may also be assessed indirectly by means of a reporter gene under the control of a promoter which is regulated by the target protein. Where binding of RIP140 to the target protein inhibits transcription of the reporter gene, disruption of the RIP140:target protein complex in the presence of a candidate compound will result in expression of the reporter gene. Conversely,
20 where binding of RIP140 to the target protein promotes transcription of the reporter gene, disruption of the RIP140:target protein interaction will inhibit transcription of the reporter gene.

The invention provides a method of screening for compounds that modulate the interaction between RIP140 and a target protein which is a transcription factor, said method comprising:

- 25 a) contacting a nucleic acid molecule, comprising a target protein-regulated promoter operatively linked to a reporter gene, with one or more candidate compounds, in the presence of RIP140 and the target protein; and
b) assessing the level of expression of the reporter gene.

- This method employs a nucleic acid molecule comprising a promoter operatively linked to a reporter gene, such that transcription of the reporter gene is under the control of the promoter and
30 is regulated by the target protein. These nucleic acids are referred to as reporter constructs.

The promoter in the construct is a target protein-regulated promoter from which the target

protein is able to promote transcription of the reporter gene. The nature of the promoter will depend on the identity of the target protein. Where the target protein is a nuclear hormone receptor, such as ER or TR, the promoter contains a hormone response element to which the nuclear hormone receptor binds to initiate transcription. SF-1 regulated promoters contain at least one gonadotroph specific element (GSE) to which SF-1 binds to initiate transcription (Bryan et al, 1999, *J. Molec. Endocrin*, 22:241-249).

The target protein-regulated promoter may be derived from the region upstream of any gene whose transcription is regulated by the target protein.

The reporter gene controlled by the promoter may be a gene which is regulated by the target protein in nature. In such situations, the reporter construct preferably includes the reporter gene and its natural upstream regulatory sequences. Preferably, however, the promoter controls transcription of a heterologous reporter gene which is easily assayed, as described above for two-hybrid methods. However, in the two-hybrid system, compounds that inhibit the interaction between that target protein and RIP140 are always detected as a result of a decrease in the expression of the reporter gene. Where the transcriptional activity of the target protein is itself detected, the result depend on whether RIP140 acts to activate or inhibit the target protein's activity: compounds that inhibit the interaction between RIP140 and the target protein give an *increase* in the expression of the reporter gene when RIP140 *inhibits* the activity of the target protein, but give a *decrease* in expression of the reporter gene when RIP140 *activates* the activity of the target protein.

For example, where the reporter gene is a fluorescent protein, inhibition of the interaction between RIP140 and a target protein that is *inhibited* by RIP140 may be detected by an *increase* in fluorescent protein expression (*cf.* two-hybrid system). In contrast, inhibition of the interaction between RIP140 and a target protein that is *activated* by RIP140 may be detected by a *decrease* in fluorescent protein expression. Similarly, where the reporter gene is a toxic gene, inhibition of the interaction between RIP140 and a target protein that is *inhibited* by RIP140 may be detected by an increase in cell *death*. In contrast, inhibition of the interaction between RIP140 and a target protein that is *activated* by RIP140 may be detected by an increase in cell *survival*.

The proteins encoded by the reporter genes may be in the form of fusion proteins as described above. For example, genes that give a visible signal may be fused downstream of a gene that is linked in nature to the promoter in the construct.

Vectors comprising reporter constructs

Reporter constructs used in the indirect screening methods of the invention may be in the form of a viral vector or a non-viral vector. Preferably, the nucleic acid molecules used in these methods of the invention are in the form of a conventional non-viral vector, such as a plasmid. Where
5 these indirect screening methods are conducted in cell-based or tissue-based assays, the introduction of the non-viral vector into the animal cells may be carried out by any method known in the art including dextran-mediated transfection, calcium phosphate precipitation, polybrene-mediated transfection, protoplast fusion, electroporation, encapsulation of polynucleotides in liposomes or direct microinjection of the DNA into nuclei, *etc.*

10 Use of nucleic acid molecules

The invention provides the use of a reporter construct, as described above, in a method of screening for compounds that modulate fat storage.

Systems for carrying out screening methods

The methods of the invention may be carried out in cell-free systems or in cells or tissues.

15 In particular, the indirect screening methods described above may be carried out in a cell-free system, in a cell or in a tissue. The cell-free system must contain all the necessary components for transcription of the reporter gene where the level of expression is detected by measuring mRNA levels, and all the necessary components for transcription and translation of the reporter gene where the level of expression is assessed by measuring protein levels.

20 It is preferred that the methods of screening of the invention be conducted in cell-free systems since this facilitates high-throughput screening of candidate compounds.

Indirect screening methods of the invention are preferably carried out in eukaryotic cells, such as mammalian (*e.g.* human) cells or tissues, or yeast cells.

25 When the indirect method of screening for compounds that inhibit the interaction of RIP140 and a target protein using a nucleic acid molecule comprising a target protein-regulated promoter is carried out in a cell, the cell should preferably express both RIP140 and the target protein endogenously. If RIP140 and the target protein are not endogenously expressed, they may be introduced into the cell using a viral or non-viral vector encoding the RIP140 or target protein. Preferably, RIP140 and the target protein are introduced into the cell in the form of plasmids.

Assessing level of expression of reporter gene

The level of expression of a reporter gene may be assessed by measuring the level of a mRNA transcribed from the reporter gene or the level of protein translated after its transcription. The measurement methods may be qualitative or quantitative.

5 Measuring level of mRNA

The level of mRNA transcribed from a reporter gene can be assessed, for example, by traditional blotting techniques described in Sambrook *et al* [*supra*]. Messenger RNA can be purified and separated using gel electrophoresis. The nucleic acids on the gel are then blotted onto a solid support, such as nitrocellulose. The solid support is exposed to a labelled probe and then washed to remove any unhybridized probe. Next, the duplexes containing the labelled probe are detected. Typically, the probe is labelled with a radioactive moiety.

Alternatively, the level of mRNA transcribed from the reporter gene may be detected by PCR-based methods. The mRNA transcribed from the reporter gene may be specifically amplified using primers that only bind to the mRNA with the amplified mRNA being detected using the blotting methods described above. The level of transcription of the reporter gene could be also detected using fluorescence resonance energy transfer (FRET) through fluorophores coupled to two oligonucleotides that are complementary to the mRNA transcribed from the reporter gene. (see Wouters *et al*, 2001, *Trends in Cell Biology* 11, 203-211).

As the cell-free system, cell or tissue will contain DNA from which the mRNA is transcribed, it is preferred to use a RNA-specific detection technique or to focus on sequence present in the mRNA transcription but not in the DNA (*e.g.* splice junctions, polyA tail *etc.*). The methods of the invention may comprise an initial step of: extracting mRNA from the cell-free system, cell or tissue; removing DNA from the cell-free system, cell or tissue; and/or disrupting DNA but not mRNA in the cell-free system, cell or tissue.

Methods for selectively extracting RNA from biological samples are well known and include methods based on guanidinium buffers, lithium chloride, acid phenol:chloroform extraction, SDS/potassium acetate, *etc.* After total RNA has been extracted, mRNA may be enriched for example using oligo-dT techniques. Methods for removing DNA from biological samples include DNase digestion. Methods for removing DNA encoding the reporter gene but not the RNA transcribed from it will use an agent which is specific to a sequence within the DNA.

Measuring level of protein

Measurement of mRNA levels is not ideal in high throughput screening methods, so it is preferred that reporter gene expression is assessed by measuring protein levels.

The level of protein expressed from the reporter gene can be conveniently measured by using an antibody which binds to the protein encoded by the reporter gene. Following removal of unbound antibody, the level of the protein encoded by the reporter gene can be determined by assessing the level of the antibody bound to it. This may be done by labelling the antibody that binds to the protein or by using a second labelled antibody which binds to the first antibody.

Where the reporter gene encodes a protein that provides a visible signal, the level of expression of the reporter gene is preferably assessed by detecting the visible signal. For example, where the reporter gene encodes a fluorescent protein such as GFP, or an enzyme such as luciferase, the level of expression may be assessed by fluorescence/luminescence detection. Where the reporter gene encodes a protein that is toxic or cytostatic, the level of expression may be assessed by looking at cell survival or cell growth.

Reference standards

A reference standard (*e.g.* a control), is typically needed in order to detect whether the interaction between the target protein and RIP140 is modulated in the method of the invention. In order to detect whether a candidate compound inhibits the interaction between the target protein and RIP140, the interaction between target protein and RIP140 in the presence of a candidate compound may be compared with the interaction between the target protein and RIP140 in the absence of a candidate compound.

The reference may have been determined before performing the method of the invention, or may be determined during (*e.g.* in parallel) or after the method has been performed. It may be an absolute standard derived from previous work.

Proteins for use in methods of the invention

The methods of the invention may use target proteins and RIP140 derived from any eukaryote. Preferably, they use target proteins and RIP140 derived from an animal, such as a mammal. Preferably, RIP140 and the target protein used in a method of the invention are both derived from the same mammal. The RIP140 and target protein are preferably both human proteins. The RIP140 gene has been cloned in a number of mammalian species including humans (Cavaillès *et al*, 1995, *EMBO J.*, 14:3741-3751) and mouse (Lee *et al*, 1998, *Mol Cell Biol*, 18: 6745-55), and

variants including natural biological variants, allelic variants and mutants containing amino acid substitutions, insertions or deletions from the wild-type sequence are in the NCBI database.

References in the methods of the invention to the use of RIP140 include these and other variants, provided that, where the method involves a RIP140/target protein interaction, the variants should retain the ability to interact with the target protein of interest. Similarly, variants of target proteins may be used if they retain the ability to interact with RIP140.

For example, if a protein has a modular structure then the methods of the invention may focus on single modules within the protein, and in particular on modules which possess a particular binding activity (e.g. on the region of ER which is involved in the ER/RIP140 interaction).

Motifs in RIP140 that interact with nuclear receptors are disclosed in WO98/49561, Lee *et al* (*Mol Cell Biol*, 1998, 18(11): 6745-44) and Wei *et al* (*J. Biol. Chem.*, 2001, 276(19): 16107-12). Thus fragments of target proteins that interact with RIP140 and fragments of RIP140 that interact with target proteins may be used in the methods of the invention. Other suitable variants of RIP140 and target proteins are known from the literature.

Polypeptides that are structurally similar to target proteins and RIP140, or to fragments of RIP140 and target proteins that retain the ability to interact, may also be used in the methods of the invention. These may be derived from natural target proteins or RIP140 or they may be prepared synthetically or using techniques of genetic engineering. In particular, synthetic molecules that are designed to mimic the tertiary structure of target proteins or RIP140 and in particular the domains of the target protein and RIP140 that interact may be used in the methods of the invention. References to the use of target proteins and RIP140 in the methods of the invention include the use of polypeptides that are structurally similar to target proteins and RIP140, or to fragments thereof.

References to the use of target proteins and RIP140 in the methods of the invention also include the use of fusion proteins comprising target proteins or RIP140, fusion proteins comprising variants or fragments thereof, or fusion proteins comprising polypeptides that are structurally similar to target proteins or RIP140 or to fragments of target proteins or RIP140. Such fusion proteins are particularly useful in two-hybrid methods.

Candidate compounds

Candidate compounds used in screening methods

Typical candidate compounds for use in all the screening methods of the invention include, but

are not restricted to, peptides, peptoids, proteins, lipids, metals, small organic molecules, RNA aptamers, antibiotics and other known pharmaceuticals, polyamines, antibodies or antibody derivatives (*e.g.* antigen-binding fragments, single chain antibodies including scFvs, *etc*), and combinations or derivatives thereof. Small organic molecules have a molecular weight of about
5 more than 50 and less than about 2,500 daltons, and most preferably between about 300 and about 800 daltons. Candidate compounds may be derived from large libraries of synthetic or natural compounds. For instance, synthetic compound libraries are commercially available from MayBridge Chemical Co. (Revillet, Cornwall, UK) or Aldrich (Milwaukee, WI). Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts may be
10 used. Additionally, candidate compounds may be synthetically produced using combinatorial chemistry either as individual compounds or as mixtures.

It has been suggested that the binding of RIP140 to nuclear receptors may be inhibited by acetylation of the domains in RIP140 that interacts with the receptor (Vo *et al*, 2001, *Mol Cell Biol*, 21(18): 6181-8). Candidate compounds may therefore be compounds identified as
15 acetylating RIP140 in a pre-screen.

Compounds identified by screening methods

The invention further provides a compound that binds to RIP140 or to a target protein, or that binds to a complex of RIP140 and a target protein, or that modulates the interaction between RIP140 and a target protein, obtained or obtainable by any of the methods described above.
20 Preferably, the compounds of the invention are organic compounds.

There is also provided a composition comprising a compound binds to RIP140 or to a target protein, or that binds to a complex of RIP140 and a target protein, or that modulates the interaction between RIP140 and a target protein, obtained or obtainable by any of the methods described above.

25 Compounds that are found to modulate fat storage may be useful for the treatment of disorders associated with deviation in fat storage in their own right or may be lead compounds for the development of new drugs for the treatment of such disorders. They may also be useful in research into the regulation of fat storage.

Pharmaceutical uses of compounds identified

30 Once a compound has been identified using one of the methods of the invention, it may be necessary to conduct further work on its pharmaceutical properties. For example, it may be

necessary to alter the compound to improve its pharmacokinetic properties or bioavailability. The invention extends to any compounds identified by the methods of the invention which have been altered to improve their pharmacokinetic properties, and to composition comprising those compounds.

- 5 The invention further provides compounds obtained or obtainable using the methods of the invention, and compositions comprising those compounds, for use as a medicament *e.g.* for increasing or decreasing fat storage. The invention also provides the use of compounds obtained or obtainable using the methods of the invention, or compositions comprising those compounds in the manufacture of a medicament to increase or decrease fat storage. The compounds may be
10 used to treat or prevent disorders associated with increased or decreased fat storage, such as obesity or anorexia. A method of increasing or decreasing fat storage comprising administering a compound obtained or obtainable by any one of the methods of the invention, or a composition comprising such a compound, to a mammal, preferably a human, is also provided.

BRIEF DESCRIPTION OF THE DRAWINGS

- 15 Figure 1: Reduction in body fat and weakening of the peritoneal wall in RIP140 null mice.
Figure 2: A) Weight gain and adipose tissue deposition in wild-type (WT) and RIP140 knockout (KO) mice; B) MRI scans and whole body spectra of wild-type and RIP140 knockout mice.
Figure 3: Food intake is normal in RIP140 null mice.
Figure 4: Comparison of glucose tolerance tests in wild-type and RIP140 null (Nrip1 null) mice.
20 Figure 5: Comparison of white adipose tissue (WAT) histology and cell size in wild-type (WT) and RIP140 knockout (KO) mice.
Figure 6: Comparison of brown adipose tissue (BAT) and skeletal muscle histology in wild-type (WT) and RIP140 knockout (KO).
Figure 7: A) Comparison of differentiation to adipocytes in wild-type (WT) and RIP140
25 knockout (KO) cells; B) Expression analysis of RIP140 mRNA.
Figure 8: Relative expression of RIP140 in different tissues of a wild-type mouse.
Figure 9: Comparison of expression of specific genes in white adipose tissue (WAT) and liver of wild-type and RIP140 null mice on different diets (st=starved; no=normal; hf=high fat). Figure 9A compares expression of PPAR γ , C/EBP α , C/EBP β and SREBP. Figure 9B compares
30 expression of Glut4, aP2, leptin and adipolectin. Figure 9C compares expression of G6Pase,

PEPCK and 24p3. Expression in wild-type mice is shown in left-hand column of each pair of columns and expression in RIP140 null mice in the right hand column of each pair of columns.

Figure 10: Expression of C/EBP α in white adipose tissue (WAT), brown adipose tissue (BAT), muscle and liver of faster and fed wild-type mice (WT) and RIP140 knockout mice (KO).

5 Expression in wild-type mice is shown in left-hand column of each pair of columns and expression in RIP140 null mice in the right hand column of each pair of columns.

Figure 11: Expression of PPAR γ in white adipose tissue (WAT), brown adipose tissue (BAT), muscle and liver of faster and fed wild-type mice (WT) and RIP140 knockout mice (KO).

10 Expression in wild-type mice is shown in left-hand column of each pair of columns and expression in RIP140 null mice in the right hand column of each pair of columns.

Figure 12: Expression of SREBP1c in white adipose tissue (WAT), brown adipose tissue (BAT), muscle and liver of faster and fed wild-type mice (WT) and RIP140 knockout mice (KO).

Expression in wild-type mice is shown in left-hand column of each pair of columns and expression in RIP140 null mice in the right hand column of each pair of columns.

15 Figure 13: Expression of Glut4 in white adipose tissue (WAT), brown adipose tissue (BAT), muscle and liver of faster and fed wild-type mice (WT) and RIP140 knockout mice (KO).

Expression in wild-type mice is shown in left-hand column of each pair of columns and expression in RIP140 null mice in the right hand column of each pair of columns.

Figure 14: Expression of leptin in white adipose tissue (WAT), brown adipose tissue (BAT), muscle and liver of faster and fed wild-type mice (WT) and RIP140 knockout mice (KO).

20 Expression in wild-type mice is shown in left-hand column of each pair of columns and expression in RIP140 null mice in the right hand column of each pair of columns.

Figure 15: Expression of CPT1b in white adipose tissue (WAT), brown adipose tissue (BAT), muscle and liver of faster and fed wild-type mice (WT) and RIP140 knockout mice (KO).

25 Expression in wild-type mice is shown in left-hand column of each pair of columns and expression in RIP140 null mice in the right hand column of each pair of columns.

Figure 16: Expression of UCP1 in white adipose tissue (WAT), brown adipose tissue (BAT), muscle and liver of faster and fed wild-type mice (WT) and RIP140 knockout mice (KO).

30 Expression in wild-type mice is shown in left-hand column of each pair of columns and expression in RIP140 null mice in the right hand column of each pair of columns.

Figure 17: Expression of PPAR α in white adipose tissue (WAT), brown adipose tissue (BAT), muscle and liver of faster and fed wild-type mice (WT) and RIP140 knockout mice (KO). Expression in wild-type mice is shown in left-hand column of each pair of columns and expression in RIP140 null mice in the right hand column of each pair of columns.

- 5 **Figure 18:** Comparison of triglyceride accumulation in liver tissue of RIP140 null mice compared to wild type when maintained on normal (a) or high fat (35%) diet (b) for 10 days.

- Figure 19:** Metabolic Phenotype of RIP140 null mice. a, Body weights of wild type (black) and RIP140 null mice (grey) fed a regular chow diet at 6 months (n=7) and 15 months (n=14 for wild type and n=11 for null). b, Magnetic resonance Imaging (MRI) and Spectroscopy (MRS) of
10 body fat content. Whole-body proton content is quantified, with fat peak indicated. Calculations from 3 representative animals revealed an approximately 70% reduction of total fat content in RIP140 null mice. c, inguinal white adipose tissue weights of wild-type (black) and RIP140 null mice (grey) at 6 months of age (n = 7 for both groups) and 15 months of age (wild-type n = 14, null n = 11). d, Morphology of inguinal white adipose tissue (WAT), brown adipose tissue
15 (BAT), and liver from wild type (WT) and RIP140 null mice (KO), fed a control chow diet. White and brown adipose tissues were stained with hematoxylin and eosin (H/E). Liver tissues were stained with Oil Red O to demonstrate lipid accumulation and counterstained with H/E. Scale bars correspond to 50 μ m. e, Increase in body weight of mice fed a high-fat diet (35% w/w) for 10 days (n = 3 for both groups). f, Serum free fatty acid levels and triglyceride levels in
20 wild type (black) and RIP140 null (grey) mice fed high fat diet for 10 days (n=6). g, Serum levels of leptin (n = 3-6) in wild type (black) or RIP140 null (grey) mice fed chow or high-fat diet. h, Morphology of inguinal white adipose tissue (WAT), brown adipose tissue (BAT), and liver from wild type (WT) and RIP140 null mice (KO), fed a high fat diet (35% w/w) for 10 days. White and brown adipose tissues were stained with hematoxylin and eosin (H/E). Liver
25 tissues were stained with oil Red O to demonstrate lipid accumulation and counterstained with H/E. Scale bars correspond to 50 μ m.

- Figure 20:** RIP140 expression in metabolic tissues and differentiated adipocytes *in vitro*. a, TaqMan real-time PCR analysis of RIP140 mRNA levels in metabolic tissues (left) and 3T3-L1 adipocytes (right), Tissue samples from white adipose tissue (WAT), brown adipose tissue
30 (BAT), skeletal muscle (Mu) and liver (Li) from wild-type mice (n = 3-7). Regulation of RIP140 mRNA levels during differentiation of 3T3-L1 adipocyte cells. Differentiation was induced in 2-day confluent cells using standard hormone cocktail of insulin, dexamethasone and

IBMX. pc (preconfluent cells). b. Oil red O staining in mouse embryo fibroblasts induced to differentiate using standard hormone cocktail as above but with addition of the PPAR agonist rosiglitazone. Cells treated identically were also subject to β -galactosidase activity analysis to demonstrate RIP140 promoter activity (right panel). Note the lack of β -galactosidase activity in wild type cells and the correlation of fat droplet containing cells staining with oil red O and β -galactosidase.

Figure 21: Upregulation of genes involved in energy dissipation in WAT a, TaqMan real-time PCR analysis of UCP1 and CPT1 gene expression in metabolic tissues from wild-type (black) and RIP140 null (grey) mice (n = 3-7). b, Immunostaining of UCP1 expression in WAT from wild type (WT) and RIP140 null (KO) mice. Scale bars correspond to 50 μ m. c, Oxygen consumption in wild type and RIP140 null mice.

Figure 22: Expression profile of adipogenic regulators and marker genes in metabolic tissues. a, b, c TaqMan real-time PCR analysis of gene expression in metabolic tissues from wild-type (black) and RIP140 null (grey) mice (n = 3-7).

Figure 23: UCP-1 gene expression is elevated in cells devoid of RIP140. a, Taqman real-time PCR analysis of UCP-1 mRNA levels in 3T3L1 and RIPKO-1 cells, pre-confluent (PC), and following treatment with standard differentiation cocktail including rosiglitazone on days 0-5 as indicated. b, Schematic representation of the murine UCP-1 enhancer element including the regulatory elements cAMP response element (CRE), peroxisome proliferator response element (PPRE), retinoid acid response element (RARE), thyroid response element (TRE), and brown adipocyte regulatory element (BRE). c, RIPKO-1 cells were transfected with UCP-1 promoter luciferase reporter constructs, as indicated, and incubated in the absence and presence of differential cocktail including rosiglitazone (D+Ro) for 1, 3 or 5 days.

Figure 24: Expression of RIP140 inhibits PPAR dependent transcription of a CPT1b promoter and a PPRE regulated reporter gene. a, PPAR α , b, PPAR δ and c, PPAR γ were analysed using a mouse CPT1 genomic fragment consisting of 2.0kb of promoter sequence cloned into the pGL3 reporter (Promega) and a 3x PPRE-TK promoter in the same vector. Transfection experiments were carried out in COS cells (PPAR α and PPAR γ) and in HEK 293 cells (PPAR δ) in the presence of specific ligands for each receptor as indicated.

MODES FOR CARRYING OUT THE INVENTION

Various aspects and embodiments of the present invention will now be described in some detail. It will be appreciated that modification of detail may be made without departing from the spirit and scope of the invention.

5 Example 1:

RIP140 knockout (Nrip1 knockout) mice were found to be viable but to have impaired growth, as demonstrated by an approximately 20-25% reduction in adult body weight. This reduction is primarily a consequence of considerably less accumulation of total body fat (approx. 75% reduction vs. wild-type), manifested in almost negligible appearance of subcutaneous fat and
10 about half the amount of inguinal fat depots (Figures 1 and 2).

Food intake was found to be similar in RIP140 knockout mice and wild-type mice, suggesting that the fat reduction in the RIP140 knockout mice was not due to a difference in food intake (Figure 3). Glucose tolerance in the RIP140 knockout mice was similar to glucose tolerance in wild-type mice (Figure 4)

15 Histological analysis of fat tissue showed that cells derived from both white adipose tissue (WAT) and brown adipose tissue (BAT) are significantly smaller in the RIP140 knockout mice (Figures 5 and 6). Despite this defect, cultured cells isolated from WAT or mouse embryos (MEFs) of RIP140 knockout mice were able to differentiate to the same degree as wild-type cells in *in vitro* differentiation assays (Figure 7A). This suggests that differentiation *per se* is not
20 impaired in RIP140 knockout mice and that leanness may result from impairment of the function of mature adipocytes. A functional role for RIP140 in mature adipocytes is supported by a dramatic up-regulation of RIP140 expression during differentiation of 3T3-L1 and MEF cells *in vitro* (Soukas *et al*, 2001, J. Biol Chem., 36: 34167-34174) and the fact that WAT display the highest expression level of all mouse tissues examined (Figure 7B and Figure 8). However, the
25 results of experiments carried out *in vitro* do not necessarily reflect the situation *in vivo* and it remains possible that lack of adipocyte differentiation is a contributing factor to the leanness observed in RIP140 knockout mice.

Detailed gene expression profile of mouse tissues revealed that the expression of a variety of genes was altered in the RIP140 null mice (Figure 9). Expression of key regulators of WAT
30 function such as C/EBP α (Figure 10), PPAR γ (Figure 11) and SREBPc (Figure 12) are all down-regulated (2- 10 times) in RIP140 knockout mice. As expected, this also leads to a decrease in target genes including Glut-4 (Figure 13) and Leptin (Figure 14).

In contrast, genes coding for two rate-limiting enzymes involved in fatty acid oxidation (CPT1b) and uncoupling protein (UCP-1) were both dramatically up-regulated (20-100 times) in RIP140 knockout WAT (Figures 15 and 16).

Since CPT1b and UCP- 1 are normally expressed at high levels in BAT, it is postulated that the WAT in RIP140 knockout mice may have adopted "BAT-like" function(s) which have led to increased fuel consumption (oxidation and uncoupling) and decreased storage in WAT of RIP140 knockout mice.

PPAR α expression was also found to be up-regulated in RIP140 knockout mice (Figure 17).

The reduction of body fat in RIP140 knockout mice appears to be due to reduced adipose cell size. Expression analysis and *in vitro* differentiation suggest that the reduced adipose cell size may be due to impaired WAT function rather than impaired adipocyte differentiation. Possible mechanism(s) for the observed phenotype is an increased fatty acid transport (up-regulation of CPT1b) and/or increased uncoupling (up-regulation of UCP- 1) in mitochondria of WAT.

Additional possible phenotypes that may affect metabolism in RIP40 knockout mice are triglyceride accumulation in muscle and protection from fat accumulation in liver after high fat feeding (Figure 18).

The experiments in Example 1 are described in more detail, along with additional results, in Example 2.

Example 2:

Results

- RIP140 null mice exhibit a lean phenotype.

RIP140 null mice are lean with a 20% reduction in body weight in both males and females compared to wild type mice, which increases with age (Fig. 19a and data not shown). Magnetic resonance imaging (MRI) and spectroscopy (MRS) of whole body fat content revealed the almost complete absence of subcutaneous fat and a marked decrease in other fat depots (Fig. 19b). Total body fat content was reduced by approximately 70% as analysed by whole body proton MRS while the weight of epididymal fat was reduced by 40-60%, according to the age of the mice (Fig. 19c). The reduction in body weight and fat content was not due to increased physical activity as judged by open field activity measurements (data not shown). In addition, food intake relative to body weight was slightly increased in RIP140 null mice (4.93 ± 0.37 and

4.70 \pm 0.20 g/mouse/day in wild type and null mice respectively) suggesting that the mechanisms controlling energy expenditure are altered in RIP140 null mice.

Histological analysis of the inguinal white fat indicates that the diameter of adipocytes was reduced from 63.2 \pm 3.7 μ m in wild-type mice to 45.2 \pm 3.5 μ m in RIP140 null mice (Fig. 19d).

5 This is equivalent to a decrease in volume of approximately 50% suggesting that the reduction in epididymal fat results from a decrease in cell size proportional to the decrease in fat content rather than to adipocyte number. The size and appearance of brown fat cells was similar in both wild type and RIP140 null mice. It is conceivable that decreased fat accumulation in WAT may have occurred in the context of a lipodystrophic phenotype, however no evidence was observed
10 of either hyperinsulinaemia, hyperglycemia (data not shown) or that fat was being stored in alternate tissues including liver, muscle and BAT, indicating that this was not the case (Fig. 19d).

The response of RIP140 null mice to the effects of feeding a high fat diet for 10 days were determined. The average weight gain in wild-type mice was 5.3 \pm 0.29 g, corresponding to a 14.5% increase of bodyweight whereas RIP140 null mice gained only 1.0 \pm 0.15 g,
15 corresponding to 3.8% of bodyweight (Fig. 19e). Serum biochemistry did not reveal increased free fatty acids and hypertriglyceridemia (Fig 19f). As expected the reduced fat content in adipose tissue leads to a reduction in circulating leptin levels (Fig. 19g). Histology (Fig.19h) and quantitative analysis of cell size in WAT indicated that there was an increase in cell diameter to 69.9 \pm 3.6 μ m in wild type mice and 50.7 \pm 1.3 μ m in RIP140 null mice. Thus the 50% decrease
20 in cell volume observed in null mice was maintained as previously noted on a normal chow diet. An increase in cell size was also observed in brown fat cells in wild type mice fed the high fat diet but not in RIP140 null mice. More importantly, oil red O staining indicated a marked increase in triglyceride accumulation in the liver of wild-type mice fed a high fat diet that was absent in RIP140 null mice (Fig. 19h), further supporting the non-lipodystrophic nature of the
25 adipose tissue alterations. This difference was also evident in mice fed a normal diet as they aged suggesting that RIP140 null mice were protected from hepatic steatosis. Thus it was concluded that RIP140 null mice are lean because they fail to store triglycerides and strikingly are resistant to high-fat diet induced obesity indicating alternative mechanisms are involved in the dissipation of excess fuel.

- 30 • Impaired adipogenesis does not account for the lean phenotype of RIP140 null mice

A survey of mouse tissues indicates that RIP140 mRNA is widely expressed, with highest levels in WAT followed by skeletal muscle and with lower levels in BAT and liver (Fig. 2a). An

expression profiling analysis of 3T3-L1 cells indicated that there was a marked progressive increase in RIP140 expression during differentiation into adipocytes (Soukas, A. *et al.*, 2001, *J Biol Chem* 276: 34167-74) and this was confirmed by quantitative real-time PCR analysis which showed that maximum levels occurred 3-6 days after induction of differentiation (Fig. 20a). Given these observations and the lean phenotype exhibited by RIP140 null mice the role of RIP140 in the differentiation and function of adipocytes was investigated.

Initially, whether RIP140 was required for adipocyte differentiation was tested by comparing the ability of fibroblasts from wild type and RIP140 null embryos to differentiate *in vitro*. It was found that confluent fibroblasts incubated in a standard hormone differentiation cocktail (with or without the PPAR γ agonist rosiglitazone) were fully capable of differentiating into adipocytes, as judged by oil red O staining, even when cells were devoid of the RIP140 gene (Fig. 20b and data not shown). Nevertheless, a link between adipocyte differentiation and RIP140 gene transcription was demonstrated by analysing the expression of the β -galactosidase gene, which has been inserted in place of the RIP140 coding sequence in the null mice (White, R. *et al.*, 2000 *Nat Med* 6: 1368-74). Fig. 20b indicates that oil red O positive cells and β -galactosidase activity were detected only in differentiated cells suggesting that the adipose phenotype is required for RIP140 promoter activity. The delay in the increase in RIP140 expression following hormone treatment and the ability of embryonic fibroblasts to differentiate in the absence of RIP140 demonstrates that adipogenesis does not depend on this nuclear receptor corepressor. The *in vivo* role of RIP140 in maintaining the function of adipose and other metabolic tissues was analysed.

- Upregulation of genes involved in energy dissipation in WAT

To investigate potential molecular mechanisms leading to defects in WAT devoid of RIP140 a detailed gene expression profile analysis was performed and in parallel studies, expression in BAT, muscle and liver was determined. Increased expression in WAT of carnitine palmitoyltransferase 1b (CPT1b; >20-fold) and the *de novo* expression of mitochondrial uncoupling protein 1 (UCP1; >100-fold) was observed; furthermore UCP-1 was also upregulated by more than 10-fold in muscle (Fig. 21a). CPT1b is necessary for the transport of free fatty acids across the outer mitochondrial membrane (McGarry, J.D. & Brown, N.F., 1997, *Eur J Biochem*, 244: 1-14; Barrero, M.J. *et al.* 2003, *Biochem J*, 369: 721-9) and together with UCP-1 is essential for thermogenesis in BAT. Immunohistochemical analysis shows the appearance of UCP1 protein in both uni-locular and multi-locular adipocytes in WAT from RIP140 null mice (Fig. 21b and data not shown). The staining for UCP1 is predominantly localised in the cytoplasmic area near the plasma membrane similar to that described in mice expressing an aP2-

Ucp1 transgene (Rossmeisl, M. *et al.*, 2002, *Eur J Biochem*, 269: 19-28). In view of the increased expression of UCP-1 in WAT from RIP140 null mice, oxygen consumption was compared *in vivo* in wild type and null mice and found an increase of 8.9% in RIP140 null mice compared with wild type animals (66.8 ± 5.1 ml/Kg/min compared to 61.3 ± 4.3 ml/Kg/min).

5 Thus upregulation of CPT1b and UCP1 in RIP140 null mice may increase mitochondrial respiration and energy uncoupling resulting in reduced storage or depletion of fat in metabolic tissues.

Many developmental, dietary, hormonal and environmental factors regulate CPT1 and UCP1 expression. Nuclear receptors, in particular the PPAR receptors, play a central role in mediating
10 many of these signalling pathways, so the expression of these receptors in metabolic tissues was examined. In all cases, expression levels were similar in wild type and RIP140 null mice with the exception of WAT where PPAR α was increased about 3-fold and PPAR γ 2 was reduced by 2-fold (Fig. 22a). A key coactivator for the activation of UCP-1 transcription is PGC-1 α , but its expression level was not elevated in RIP140 null mice (Fig. 22b). Similarly, the levels of SRC1
15 and TIF2, which have been found to control energy balance between white and brown adipose tissue, were not altered (Fig. 22b). In addition to PPAR γ 2 the expression of certain transcription factors required for adipocyte function, such as CEBP α and SREBP1c, were reduced by 50-60% in WAT from RIP140 null mice but not in other tissues (data not shown). Consistent with these reductions, there was a decrease in the expression of corresponding target genes including for
20 example, adiponectin, leptin, glucose transporter 4 (Glut4) and aP2, (Fig. 22c, Fig. 19g and data not shown).

- RIP140 corepressor targets the UCP1 enhancer element.

The mechanism by which RIP140 may regulate gene expression was then investigated by examining the promoters for the UCP1 and CPT1 genes in transfected cell lines. To perform
25 these experiments a RIP140 null cell line, termed RIPKO-1 cells, was generated from mouse embryonic fibroblasts that could be induced to undergo adipocyte differentiation *in vitro* as judged by its ability to express a number of marker genes and to accumulate triglyceride (data not shown). Importantly, in contrast to 3T3L1 cells, treatment of confluent RIPKO-1 cells with hormone cocktail to induce adipocyte differentiation results in the induction of UCP1 (Fig. 23a)
30 and increased expression of CPT1 (data not shown). The time course of this derepression correlates with the increase in RIP140 expression observed in 3T3L1 cells induced to

differentiate (Fig. 20a) which combined with the altered *in vivo* expression is consistent with a mechanistic link between corepressor function and the UCP1/CPT1 gene regulation.

To analyse whether the increase in UCP1 expression reflects an alteration in gene transcription two luciferase reporter genes under the control of UCP1 promoter sequences were generated.

5 One contained 4 kb of DNA upstream of the start site of transcription and the other a 220 bp fragment previously shown to contain multiple regulatory elements for nuclear receptors (Fig. 23b) (Lowell, B.B. & Spiegelman, B.M., 2000, *Nature* 404: 652-60; Sears, I.B., *et al*, 1996, *Mol Cell Biol*, 16: 3410-9; Larose, M. *et al.*, 1996, *J Biol Chem* 271: 31533-42; del Mar Gonzalez-Barroso, M. *et al.*, 2000, *J Biol Chem*, 275: 31722-32 (2000). The activation of both reporter
10 genes was apparent in RIPKO-1 cells only when they had undergone adipocyte differentiation such that the increase in promoter activity paralleled the increase in UCP1 mRNA levels (compare Figs. 23a and 23c). Thus the absence of RIP140, together with the action of one or more factors induced upon adipocyte differentiation, permits the transcription of the UCP1 gene. The effect of re-expressing RIP140 in RIPKO-1 cells was examined and it was found that it
15 markedly reduced transcription from both UCP1-luciferase reporter genes (Fig. 23c), consistent with its function as a transcriptional corepressor. Thus, it was concluded that UCP1 transcription is subject to suppression by RIP140 and that this is mediated by the 220bp regulatory region found 2.5 kb upstream of the start site of transcription, presumably by targeting transcription factors that bind to elements within this region of the promoter.

20 The UCP-1 enhancer element has been shown to be a target for a number of nuclear receptor signalling pathways, the best characterised of which is PPAR α . On the other hand, the lean phenotype of RIP140 null mice and the level of upregulation of UCP1 resembles that found in mice in which an activated form of PPAR δ is overexpressed in adipose tissue, raising the possibility that this PPAR isoform might also be a target for repression. The ability of RIP140 to
25 repress the transcriptional activity of the three individual PPAR isoforms in cells devoid of these receptors was examined. Since the UCP1 promoter exhibited minimal activity in heterologous cell lines compared with that in adipocytes, luciferase reporter genes containing the CPT1 promoter sequences and a PPRE consensus sequences as a control were analysed. It was found that RIP140 was able to repress the ability of PPAR α (Fig. 24a) PPAR δ (Fig. 24b) and PPAR γ
30 (Fig. 24c) to stimulate transcription from both the CPT1b promoter and the PPRE containing reporter gene. This redundancy between PPAR α and PPAR δ for ligand dependent expression of CPT1b is in agreement with previous studies (Gilde, A.J. *et al.*, 2003, *Circ Res* 92: 518-24). Thus PPARs are potential targets for repression by RIP140 so that the absence of this

corepressor may increase their transcriptional activity and consequently lead to up-regulation of genes such as CPT1b and UCP1.

Discussion

Previous work has established the importance of transcriptional activation by nuclear receptors in both the control of adipogenesis and the regulation of energy balance by thermogenesis, with PPARs playing a central role, *e.g.* there is substantial evidence that PGC1 α is a key transcriptional coactivator and metabolic regulator in BAT, muscle and liver. This coactivator plays a crucial role in integrating cellular signals controlling energy balance and nutrient homeostasis by the coordination and induction of gene expression in a cell type and tissue specific manner. Recent studies also suggest a role for PGC1 α on the action of ERR α in adipose tissue and muscle, while the balance between SRC1, TIF2 and PGC1 α appears to modulate energy homeostasis between white and brown adipose tissue compartments.

In contrast, the present work demonstrates an essential role for the corepressor RIP140 in the maintenance of energy homeostasis with ligand dependent transcriptional repression of nuclear receptors playing a fundamental role in preventing the expression of key metabolic genes in WAT. The upregulation of UCP1 is an intrinsic property of WAT devoid of RIP140 since this was also observed when null cells were differentiated into adipocytes in cell culture. It is proposed that the UCP1 gene is normally repressed in WAT as a consequence of RIP140 targeting a 220 bp enhancer element 2.5 kb upstream of the start site of transcription. In BAT the UCP1 enhancer may be regulated by all three subtypes of PPAR, by retinoids and thyroid hormone, as well as through activation of β -adrenergic receptor intracellular signalling pathways. Expression of UCP1 has been shown to be induced in WAT in transgenic mice by introduction of an activated form of PPAR δ and also in human white adipocytes in culture as a result of expression of PGC1 α and activation by ligands for PPAR γ (Tiraby, C. *et al.*, 2003, *J Biol Chem*, 278: 33370-6). RIP140 is capable of interacting with all of these receptors in a ligand-dependent manner and of repressing their transcriptional activity, as shown for PPARs in this study (Cavaillès, V. *et al.*, 1995, *Embo J*, 14: 3741-51; L'Horsset, F. *et al.*, 1996, *Mol Cell Biol*, 16: 6029-36; Treuter, E. *et al.*, 1998, *Mol Endocrinol* 12: 864-81; Tazawa, H. *et al.*, 2003, *Mol Cell Biol*, 23: 4187-98).

Recent studies have demonstrated that RIP140 has intrinsic repressive activity contained within four repression domains within the protein Vo, N. *et al.*, 2001, *Mol Cell Biol*, 21: 6181-8; Kumar, V. *et al.*, 2002, *Mol Cell*, 10: 857-69; data not shown). The temporal and cell type specific

control of RIP140 expression provides an additional level of regulation of repressor function. For example, the delayed onset of RIP140 expression may be necessary to avoid interference with the action of PPAR γ during the process of adipogenesis while facilitating a role in regulating the activity of the differentiated adipocyte. Clearly RIP140 is essential to prevent the upregulation of genes involved in energy dissipation and mitochondrial uncoupling, processes that compromise the function of the adipocyte as a site of energy storage in the form of triglycerides.

In conclusion, these observations show that RIP140 plays a crucial role in energy homeostasis and demonstrate a novel role for corepressor action in addition to coactivator recruitment in determining adipocyte function. Since the interaction of RIP140 with nuclear receptors is a ligand-dependent process, specific recruitment of this corepressor provides a novel therapeutic target for the treatment of obesity and related disorders.

Materials and methods

Animals

The generation of RIP140 null mice have previously been described (White, R. *et al.*, 2000, *Nat Med*, 6: 1368-74). Mice used in this study were backcrossed 6 generations to C57BL/6J background. Mice were maintained under standard conditions, with controlled light and temperature and fed a chow diet *ad libitum*, except for high fat diet experiments where mice were fed a 35% w/w diet (Lillico). All experiments were performed according to Home Office guidelines.

Magnetic resonance imaging (MRI) and spectroscopy (MRS)

Mice were scanned using a 4.7T Varian system (Palo Alto, USA). Whole body images (between 40-45 slices; 2 mm thick) were obtained for each mouse using a spin-echo sequence (TR4500/TE20). Whole body spectra were obtained using a TR=10 s. Localised proton spectra of liver and muscle were obtained from a 3x3x3 mm voxel using a PRESS sequence (TR10000/TE14).

Serum analysis

Leptin measurements were determined using Linco Research Inc Mouse Leptin RIA kit. Triglycerides were measured using Triglyceride GPO-Trinder reagent (Sigma) and free fatty acids determined using ADIFAB free fatty acid indicator (Molecular Probes). All assays were carried out according to the manufacturers protocols.

Morphological and immuno-histochemical analysis

Tissues were fixed in neutral buffered formalin, embedded in paraffin and sectioned at 5 µm onto poly-L-lysine coated slides. For histology, sections were stained with haematoxylin and eosin. For immunohistochemistry, deparaffinised sections were incubated in 0.3% hydrogen peroxide (Sigma) in methanol for 30 min to inactivate endogenous peroxidase, rinsed in PBS and incubated in 1:75 normal goat serum/PBS for 30 min to reduce non-specific background staining. Sections were incubated overnight at 4°C with polyclonal rabbit anti-mouse primary antibody to UCP1 (AB3038, Chemicon International) diluted 1:800 in PBS. Primary antibody was detected using the Vectastain Elite ABC kit (Vector Laboratories), with enzymatic detection using 0.25 mg/ml diaminobenzidine (Sigma) and 0.06% hydrogen peroxide in PBS. Sections were counterstained with haematoxylin. Oil Red O: Frozen sections (10 µm) of formalin-fixed liver were mounted on poly-L-lysine coated slides and stained with Oil Red O (0.15% in 60% isopropanol) for 5 min. Sections were counterstained with haematoxylin and mounted in glycerol gelatin (Sigma).

Oxygen consumption

Oxygen consumption was measured in wild type and RIP140 null mice fed a chow diet (7 mice per group) using the OXYMAX System v4.66 (Columbus Instruments, Columbus, OH) and conditions of a settling time of 180 s, measuring time of 60 s, and room air as a reference. Animals were placed individually in 4 0.3-liter chambers. Results are expressed as ml/kg/min.

Cell culture of mouse embryonic fibroblasts and 3T3-L1 cells.

Mouse embryonic fibroblasts (MEFs) were isolated and cultured using standard protocol in DMEM/F12 media supplemented with 10% foetal bovine serum. For differentiation experiments, MEFs cultured for 4-6 passages were used. 3T3-L1 cells were cultured in DMEM/F12 media supplemented with 10% newborn bovine serum. Differentiation of 2 day post-confluent MEFs and 3T3-L1 cells was performed as previously described (Soukas, A. *et al*, 2001, *J Biol Chem* 276: 34167-74) with the modification that MEFs which were also supplemented with rosiglitazone. Differentiated cells were visualised with oil red O staining. β-galactosidase activity was analysed as previously described (White, R. *et al.*, 2000, *Nat Med*, 6: 1368-74).

The cell line RIPKO-1 was generated by culturing RIP140 null MEFs for greater than 20 passages. UCP-1 promoter luciferase reporter constructs were generated by cloning a 4Kb fragment or 220bp enhancer element (-2530 to -2310 bp relative to ATG) of the 5' flanking

region of the murine UCP-1 gene into pGL3/basic vector. RIPKO-1 cells were transfected, immediately following plating into 24-well plates, using Fugene6 with 1µg reporter gene, 250ng pRL-CMV and/or 500ng pCI-RIP. Twenty-four hours following transfection the medium was replenished with either control medium or medium containing differentiation cocktail. Cells were harvested for luciferase assay 1, 3, and 5 days following the addition of the differentiation cocktail.

Expression analysis

Total RNA was isolated using TRIzol according to the manufacturer's instructions. To obtain first strand cDNA for further analysis, 1 µg total RNA was treated with deoxyribonuclease, and cDNA was prepared using the Superscript First-Strand Synthesis System for RT-PCR according to the manufacturer's instructions. Real-time PCR was performed with the ABI PRISM 7700 Sequence Detection System. RIP140 and L19 expression was determined using specific primers and TaqMan probes. Expression of all other genes was determined with SYBR-green reagent using specific primers according to the manufacturer's instructions. Expression levels for all genes were correlated to mean value of two independent internal controls, the ribosomal coding gene L19 and cyclophilin. Primer and probe sequences are available on request.

Transient transfection

Cells were routinely maintained in DMEM supplemented with 10% fetal bovine serum. Twenty-four hours before transfection, cells were plated in 96-well microtiter plates in phenol red-free medium supplemented with 5% dextran charcoal-stripped serum. HEK293 or Cos7 cells were transfected using FuGENE 6 with 20ng luciferase reporter, 5ng pRLCMV control, 0.5ng pcDNA3 PPARα, δ or γ and/or 10ng pCI RIP140 where indicated. 24h after transfection cells were treated with ligands WY14,643 (10µM), Rosiglitazone (5µM), or GW501516 (5nM). Cells were harvested 24hr after addition of ligands and luciferase activity measured using a Victor 2 luminometer. The reporter firefly luciferase activity was measured using the LucLite™ kit, subsequently the *Renilla* luciferase activity used as internal control was determined by the addition of EDTA (8 mM final concentration) and Coelenterazine substrate (4.7 µM (250ng/well)) to the firefly luciferase reaction. The *Renilla* luciferase activity was used to correct for differences in transfection efficiency.

CLAIMS

1. A method for screening for a compound that modulate fat storage in a mammal, comprising one of the following three steps:

- (i) identifying a compound which binds to RIP140 or to a RIP140 target protein;
- 5 (ii) identifying a compound which binds to a complex of RIP140 and a RIP140 target protein; or
- (iii) identifying a compound which modulates the binding interaction between RIP140 and a RIP140 target protein,

and further comprising the step of administering to a mammal a candidate compound identified in step (i), (ii) or (iii) and assessing its effect on fat storage in the mammal.

2. A method according to claim 1, wherein step (iii) comprises:

- a) mixing RIP140, the target protein and one or more candidate compounds;
- b) incubating the mixture to allow RIP140, the target protein and the candidate compound(s) to interact; and
- 15 c) assessing whether interaction between RIP140 and the target protein is modulated.

3. A method according to claim 2 comprising:

- a) contacting a cell containing a nucleic acid molecule comprising a promoter operatively linked to a reporter gene with: (i) a first fusion protein comprising one of RIP140 and a target protein fused to the activation domain of a transcription factor,
- 20 (ii) a second fusion protein comprising the other of RIP140 and a target protein fused to the DNA-binding domain of a transcription factor; and (iii) a candidate compound; and
- b) assessing the level of expression of the reporter gene,

wherein interaction between RIP140 and the target protein promotes transcription of the reporter gene by activating said promoter.

4. A method according to claim 1 wherein step (iii) comprises:

- a) contacting a nucleic acid molecule, comprising a target protein-regulated promoter operatively linked to a reporter gene, with one or more candidate compound(s) in the presence of said target protein and RIP140; and
- 30 b) assessing the level of expression of the reporter gene.

5. A method according to any one of claims 2 to 4, wherein the promoter controls transcription of a reporter gene with which it is linked in nature.
6. A method according to any one of claims 2 to 5, wherein expression of the reporter gene gives a detectable signal.
- 5 7. A method according to claim 6, wherein the reporter gene encodes a fluorescent protein, an enzyme, a toxic protein or cystostatic protein.
8. A method according to any preceding claim, wherein said method is carried out in a cell free system, a cell or a tissue.
9. A method according to any one of claims 2 to 8, wherein the nucleic acid molecule is in the
10 form of a non-viral vector.
10. A method according to any one of claims 2 to 9, wherein the step of assessing the level of expression of the reporter gene comprises measuring the level of mRNA transcribed from the reporter gene.
11. A method according to any one of claims 2 to 9, wherein the step of assessing the level of
15 expression of the reporter gene comprises measuring the level of protein translated after transcription of the reporter gene.
12. A compound that binds to RIP140 or to a target protein, obtained or obtainable by a method of claim 1.
13. A compound that binds to a complex of RIP140 and a target protein, obtained or obtainable
20 by a method of claim 1.
14. A compound that modulates the interaction between RIP140 and a target protein, obtained or obtainable by a method of any preceding claim.
15. A method according to any one of claims 1 to 11, or a compound according to any one of claims 12 to 14, wherein the target protein is selected from AhR, ER, RAR, TR, RXR, VDR, PPAR, SF-1 and DAX-1.
25
16. A method or a compound according to claim 15, wherein the target protein is selected from PPAR α , PPAR γ and PPAR δ .
17. A method of assessing the effect of a compound according to any one of claims 12 to 14 on
30 fat storage, comprising administering the compound to a mammal and assessing its effect on fat storage.

18. A pharmaceutical composition comprising a compound according to any one of claims 12 to 14.
19. A compound according to any one of claims 12 to 14 for use as a medicament.
20. Use of a compound according to any one of claims 12 to 14 in the manufacture of a
5 medicament for treating or preventing a disorder associated with increased or decreased fat storage.
21. Use according to claim 19 wherein said disorder is obesity or anorexia.
22. A method of altering fat storage in a mammal, comprising administering a compound according to any one of claims 12 to 14, or a composition according to claim 17.

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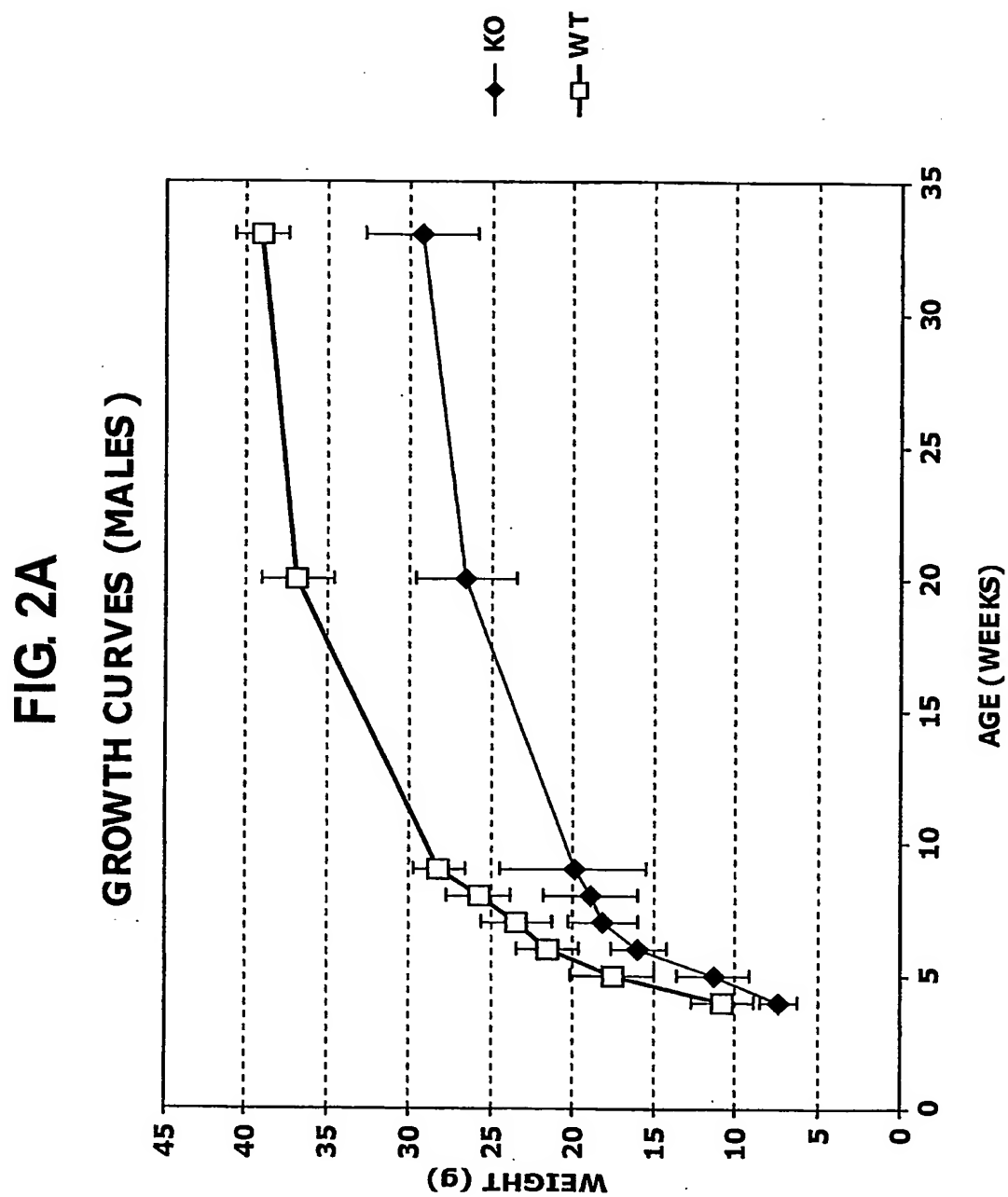
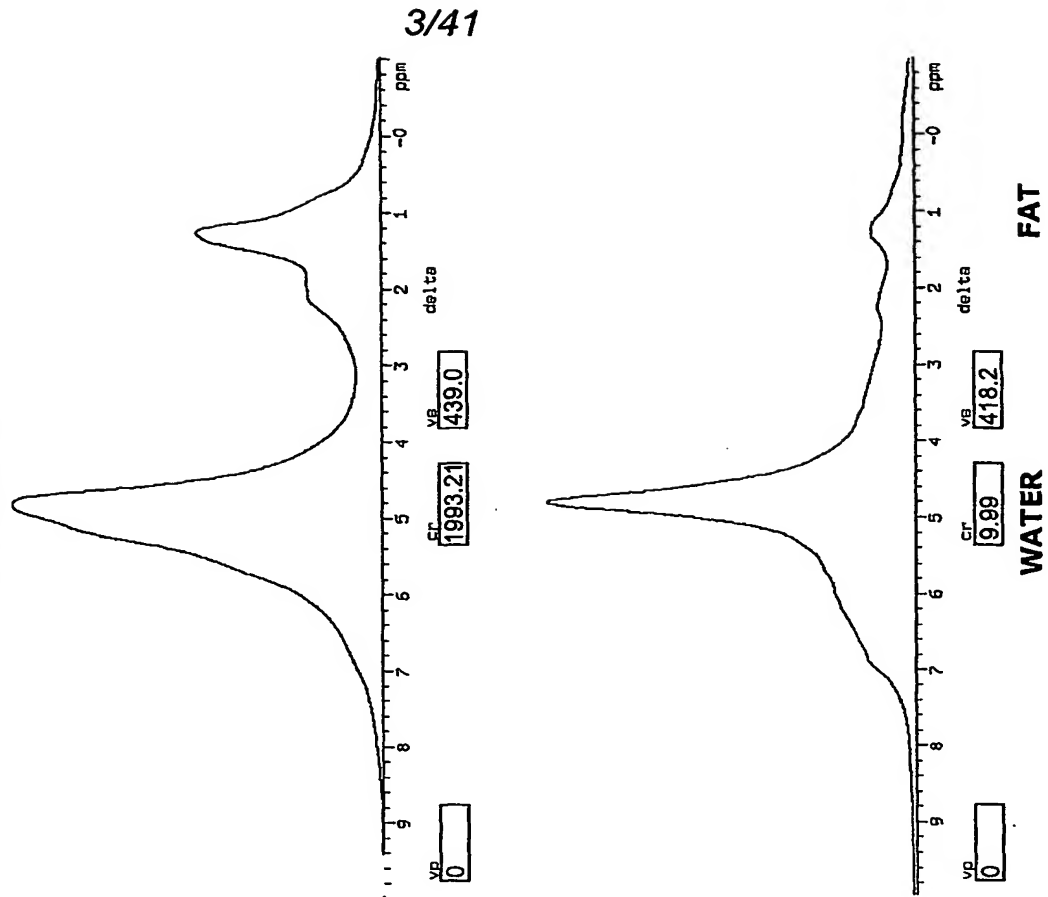
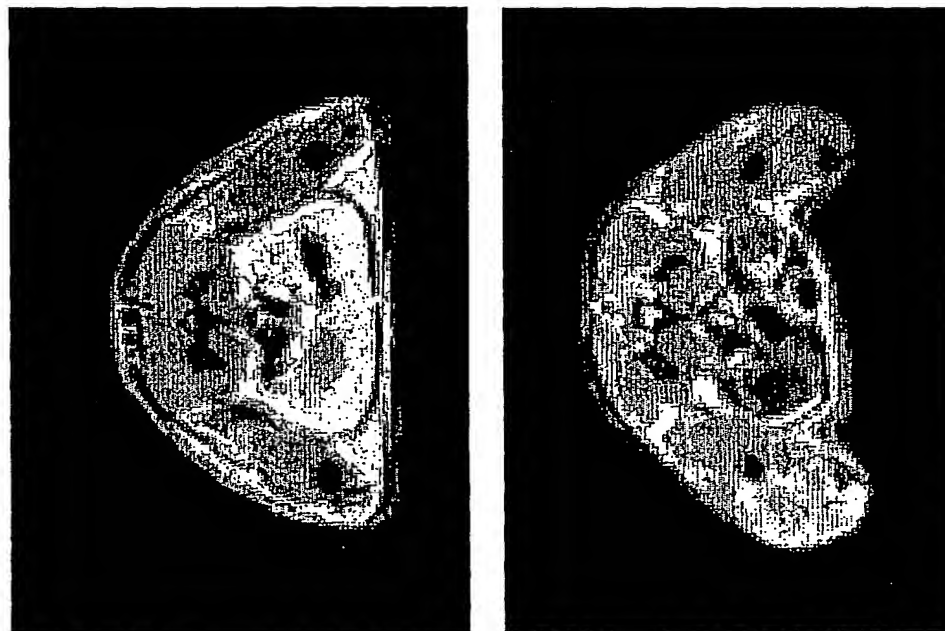
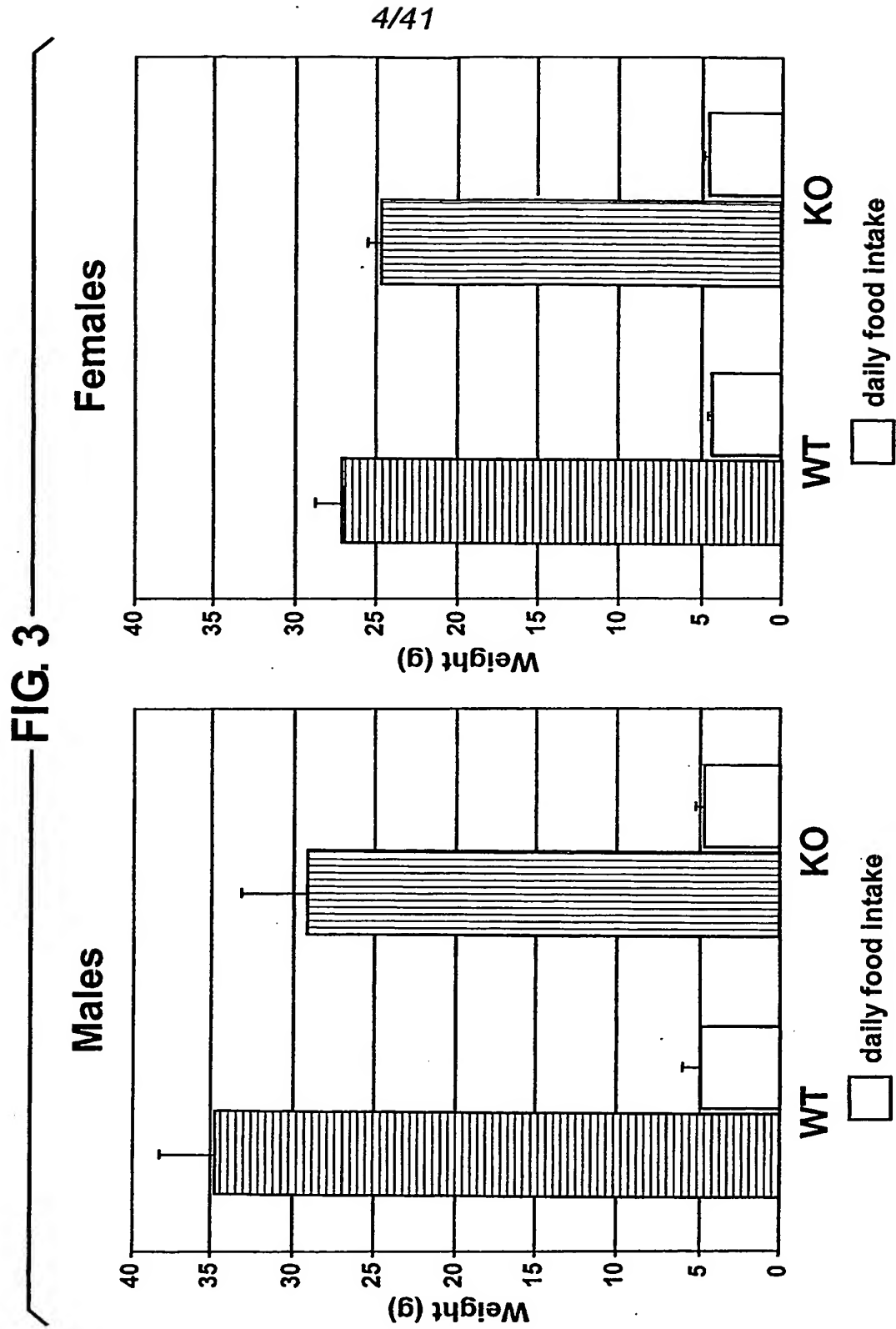
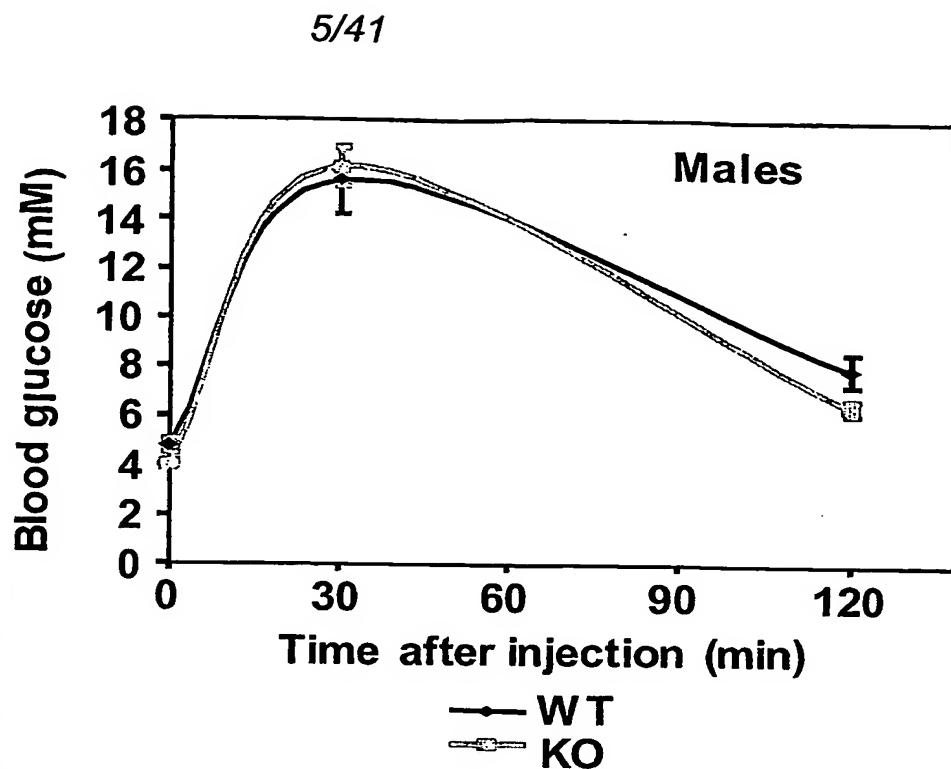
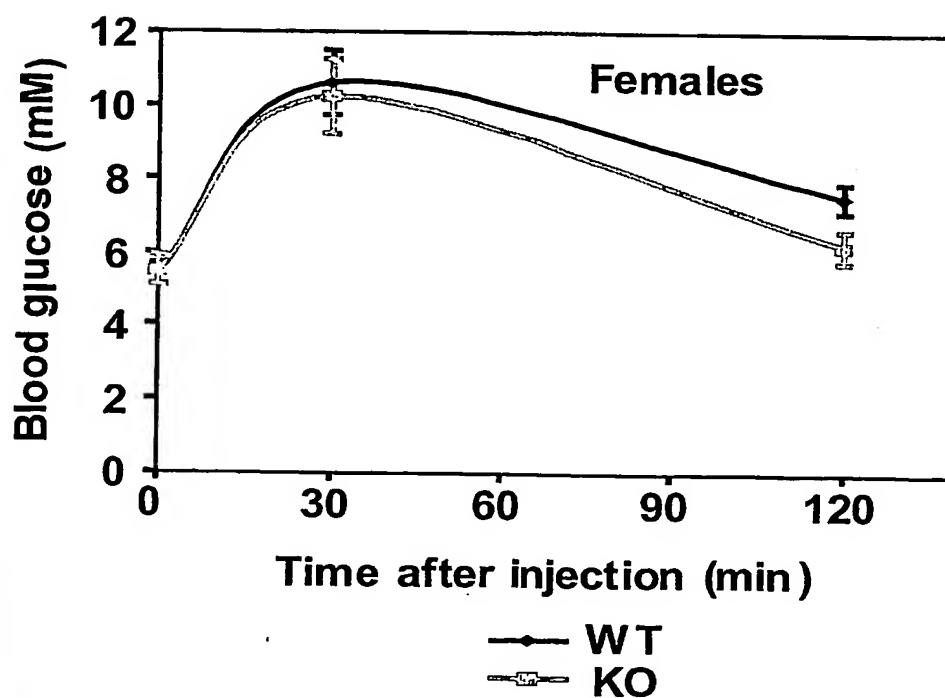


FIG. 2B





**FIG. 4**

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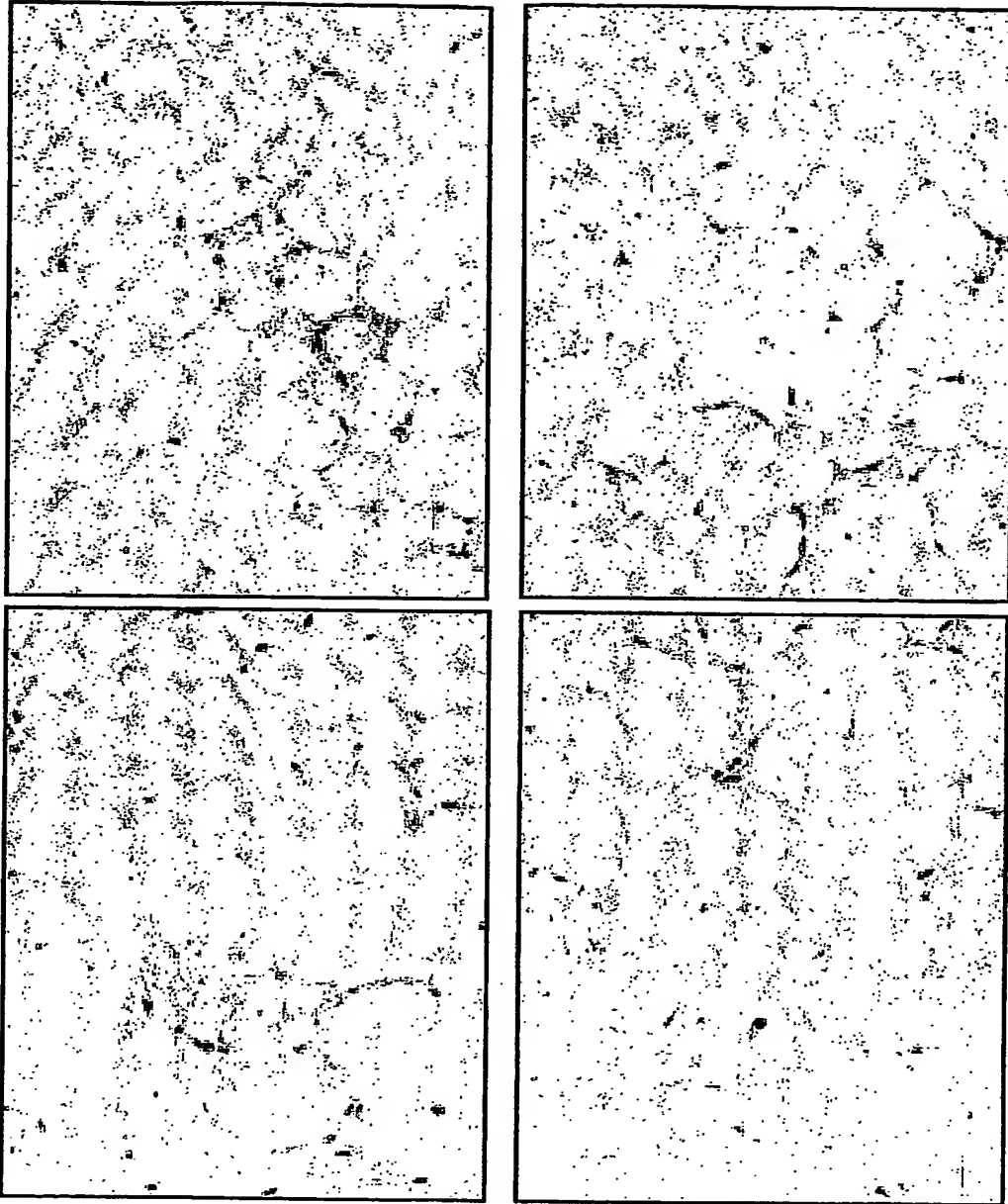
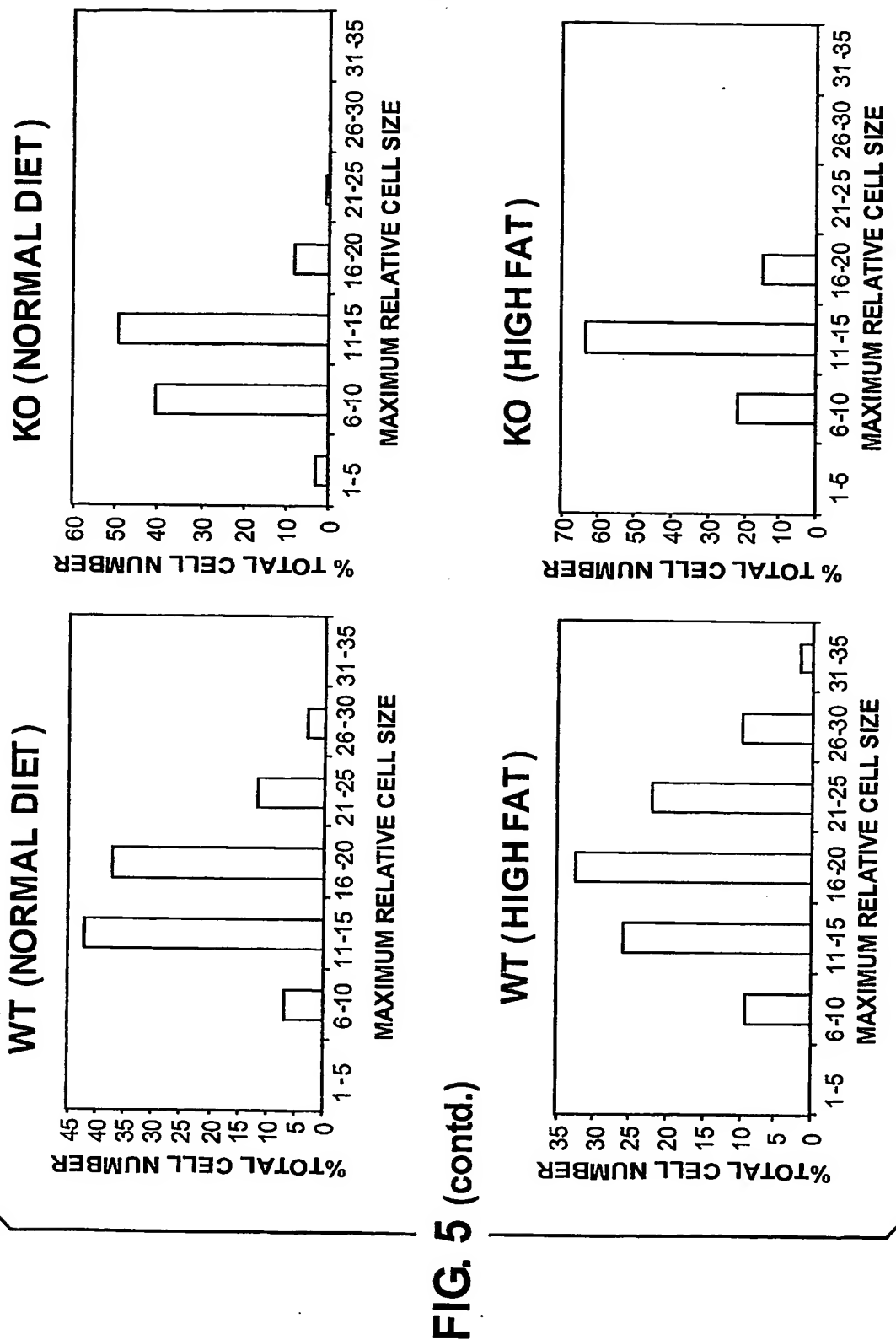


FIG. 5

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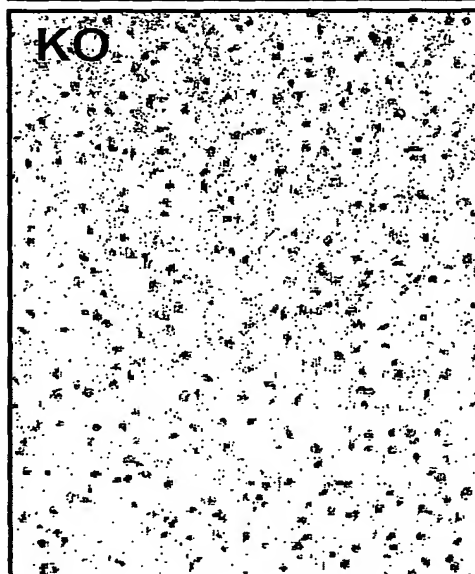
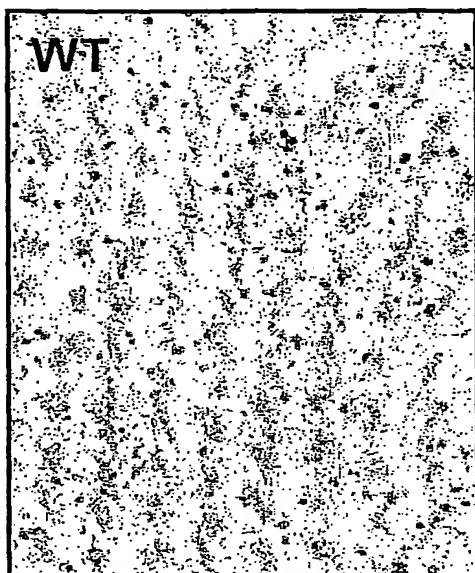
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FIG. 6

Histology of BAT
from mice
maintained for 10
days on a 35%
(HIGH) fat diet.



Histology of
skeletal muscle
from mice fasted
for 18 hours

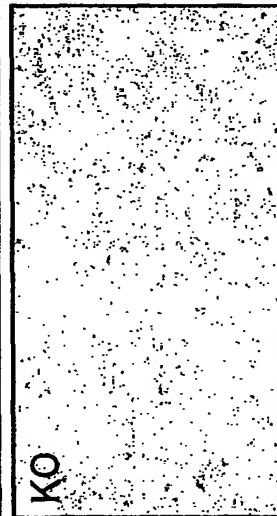
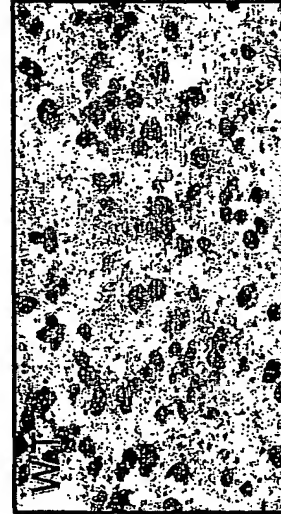
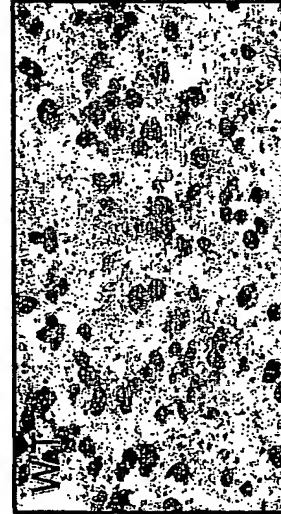
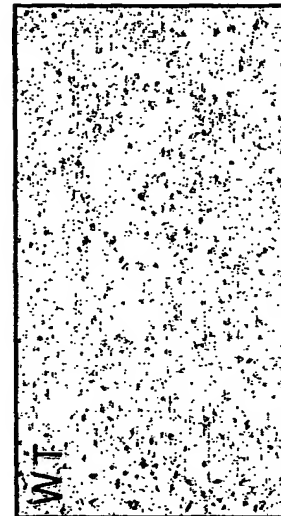


FIG. 7A

Nrip1/RIP140 null cell differentiation to adipocytes in vitro is unaffected

Untreated

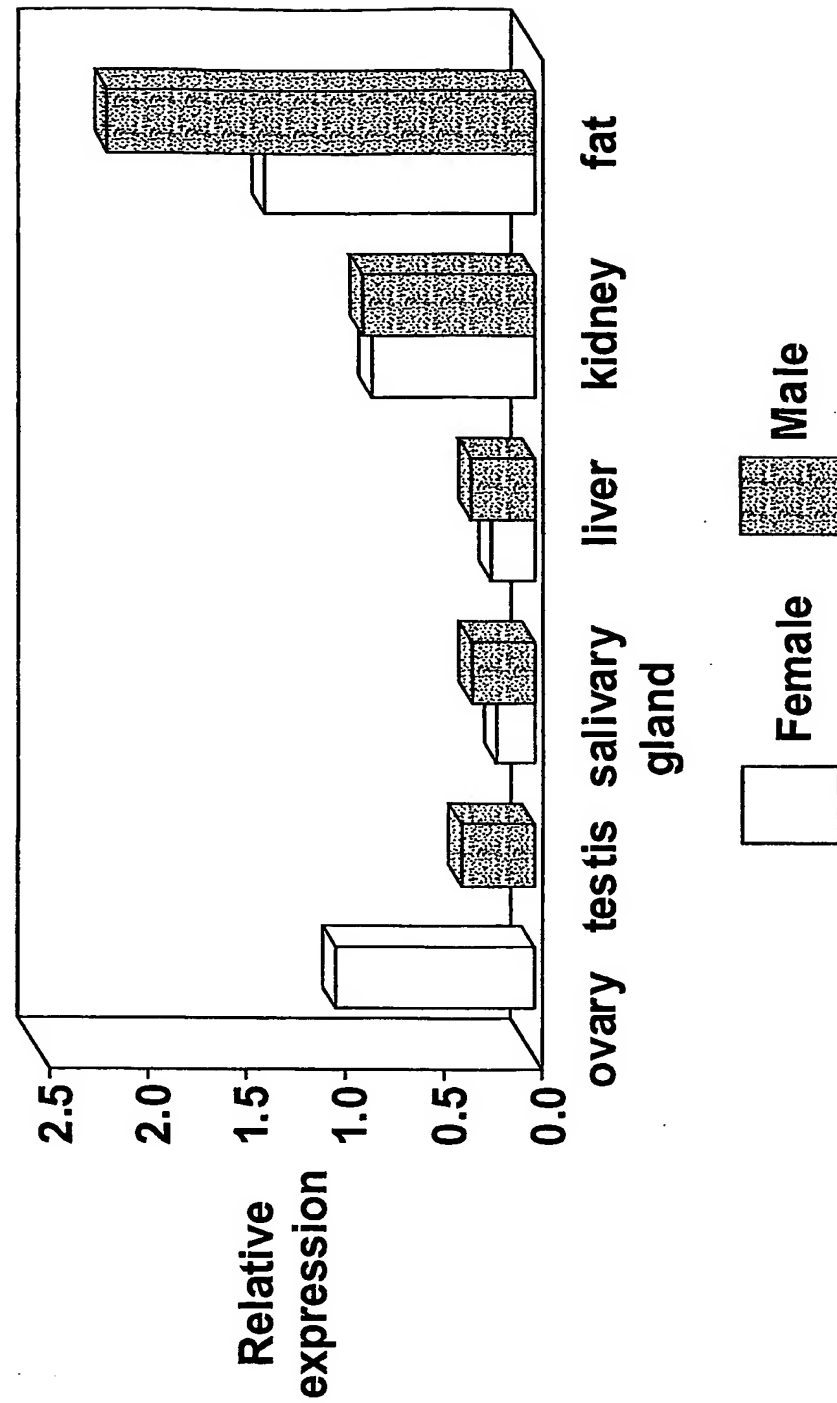
Differentiation + Rosiglitazone (8 days)



Oil-Red-O staining and b-gal activity in WT and KO MEF cells

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FIG. 7B
Expression analysis of Nrip1 mRNA



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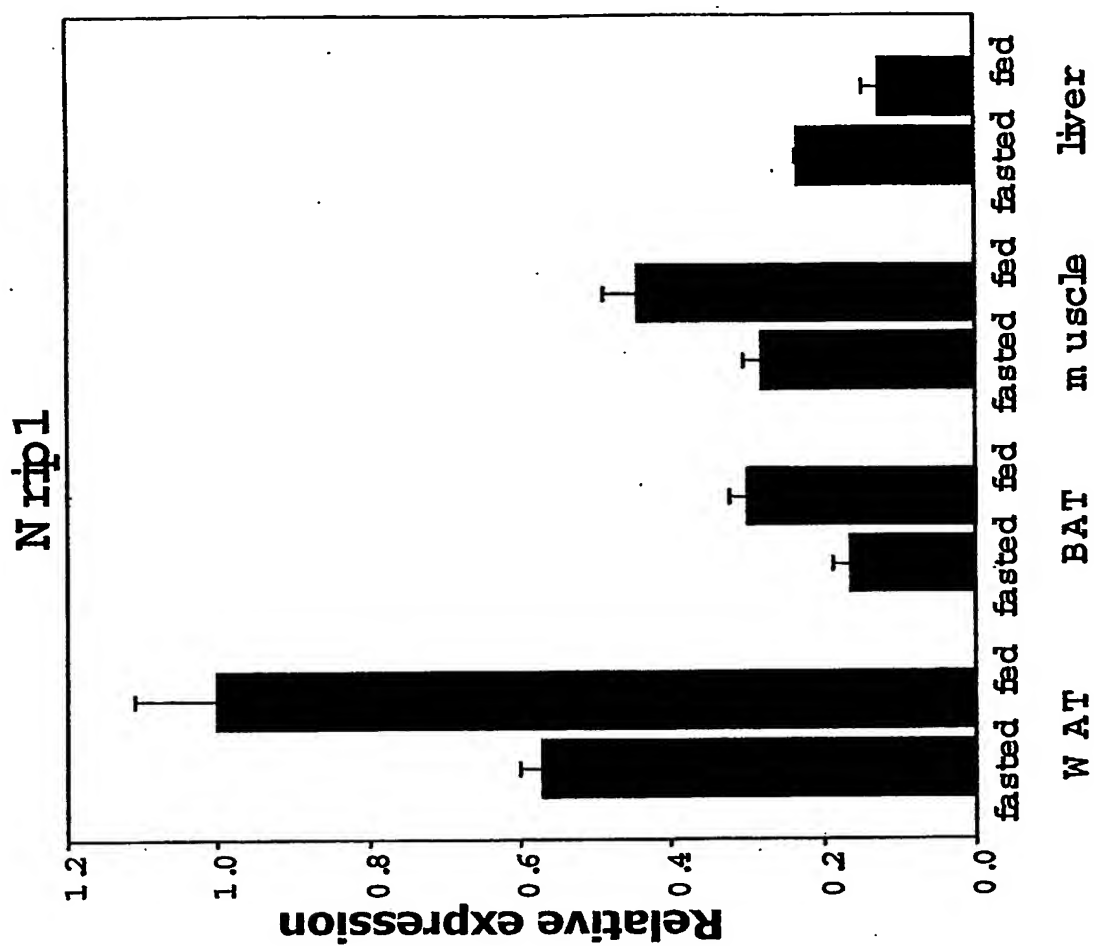
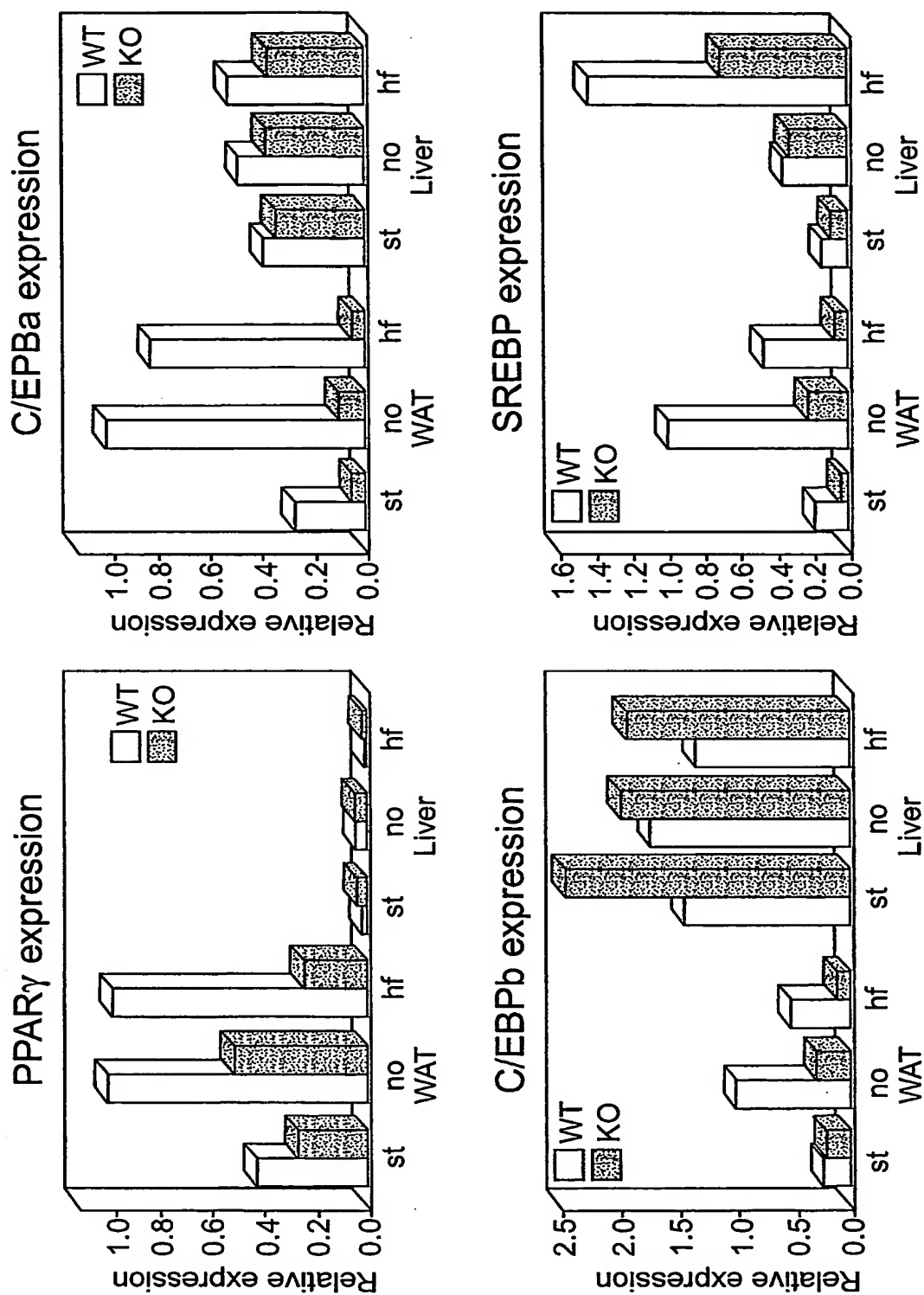


FIG. 8

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FIG. 9A



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FIG. 9B

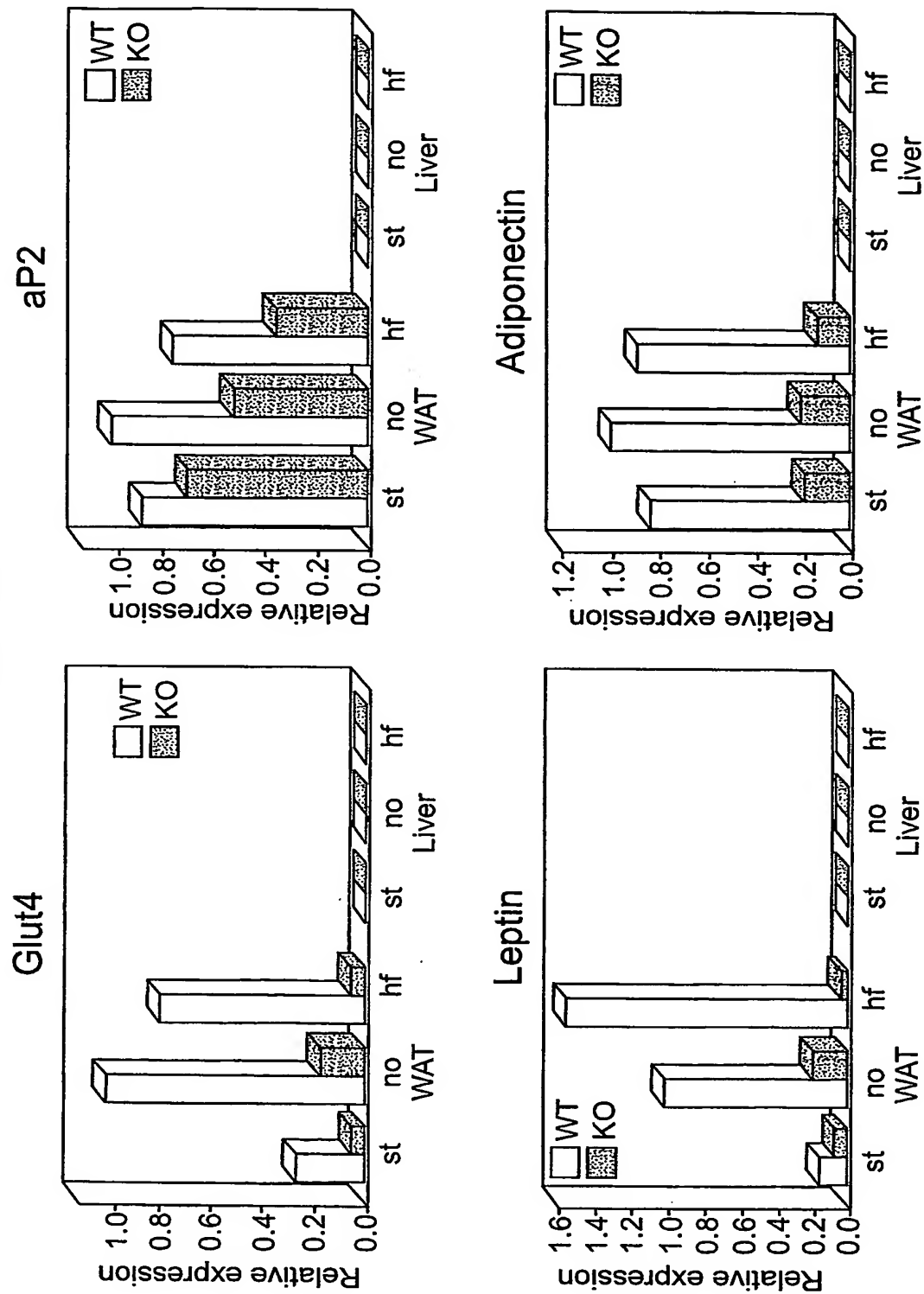


FIG. 9C

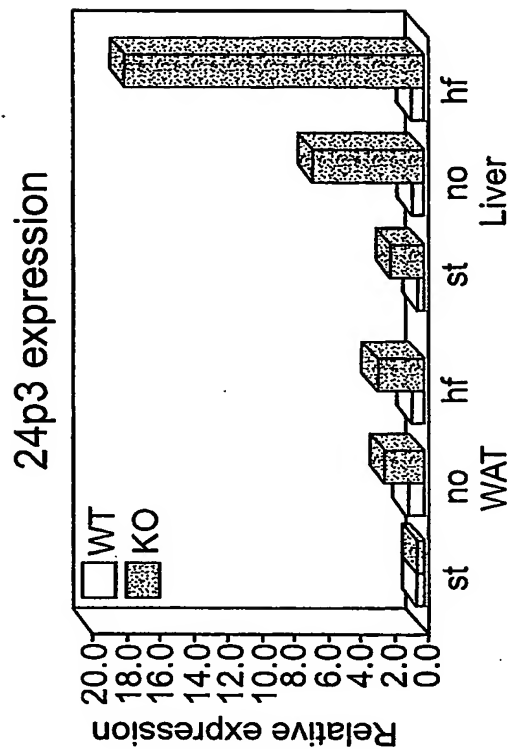
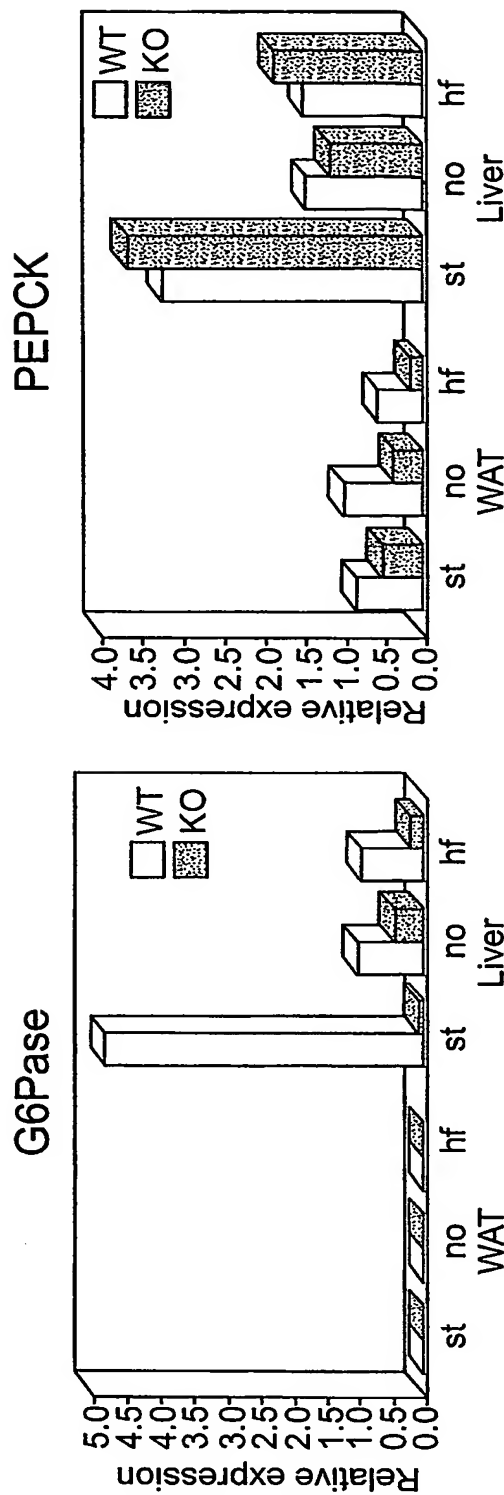
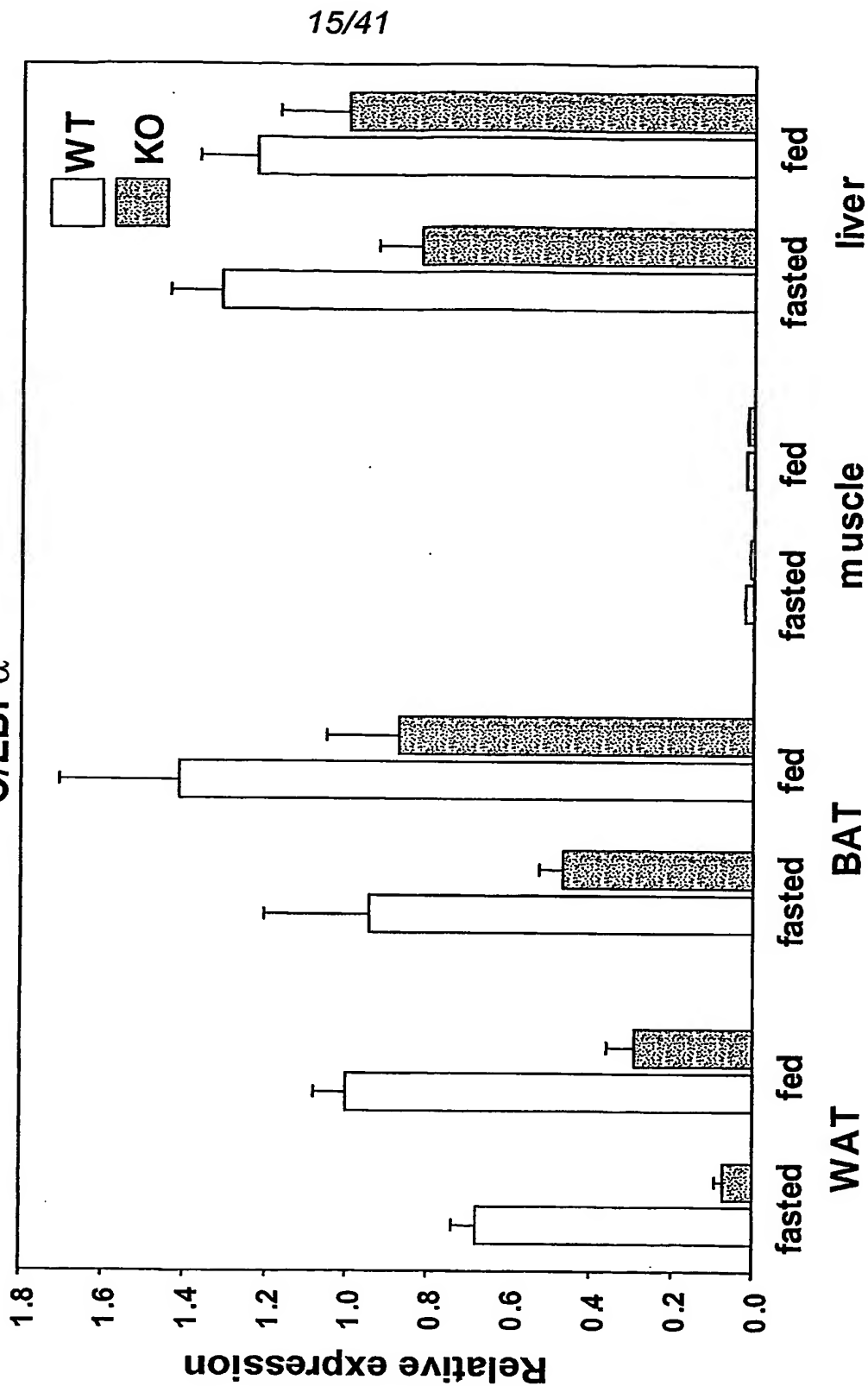


FIG. 10

C/EBP α



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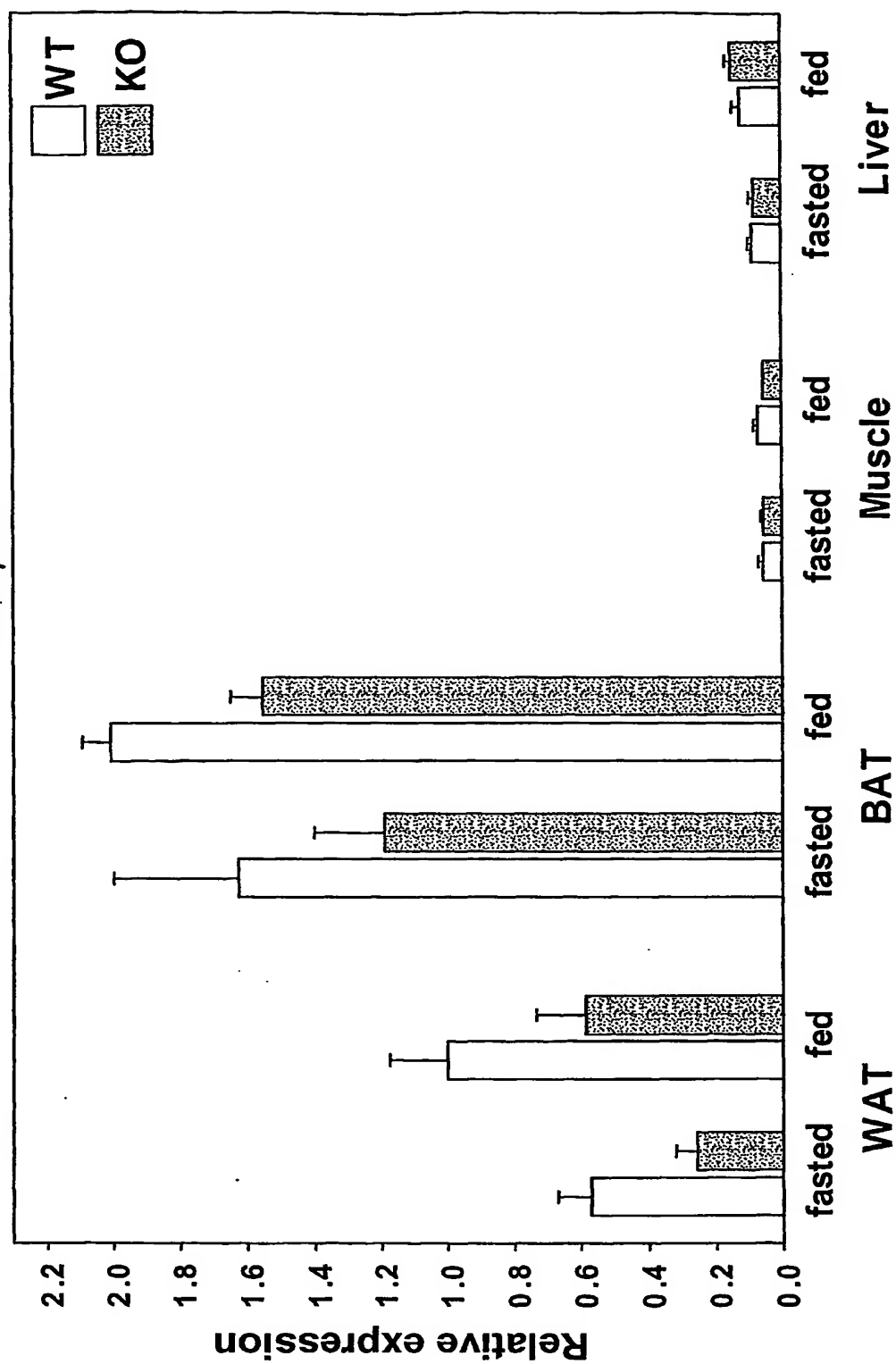
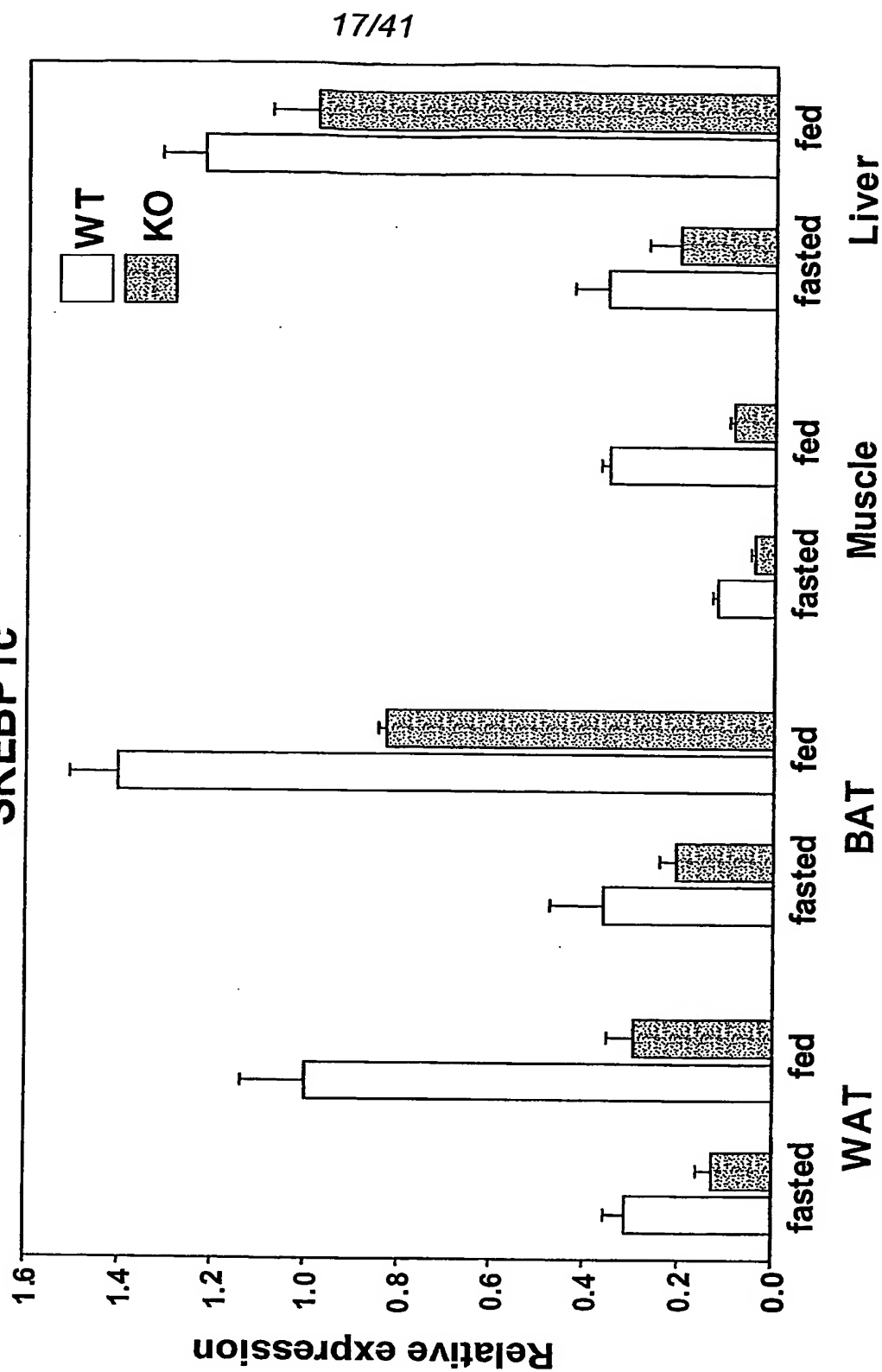
FIG. 11
PPAR γ 

FIG. 12
SREBP1c



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FIG. 13
Glut 4

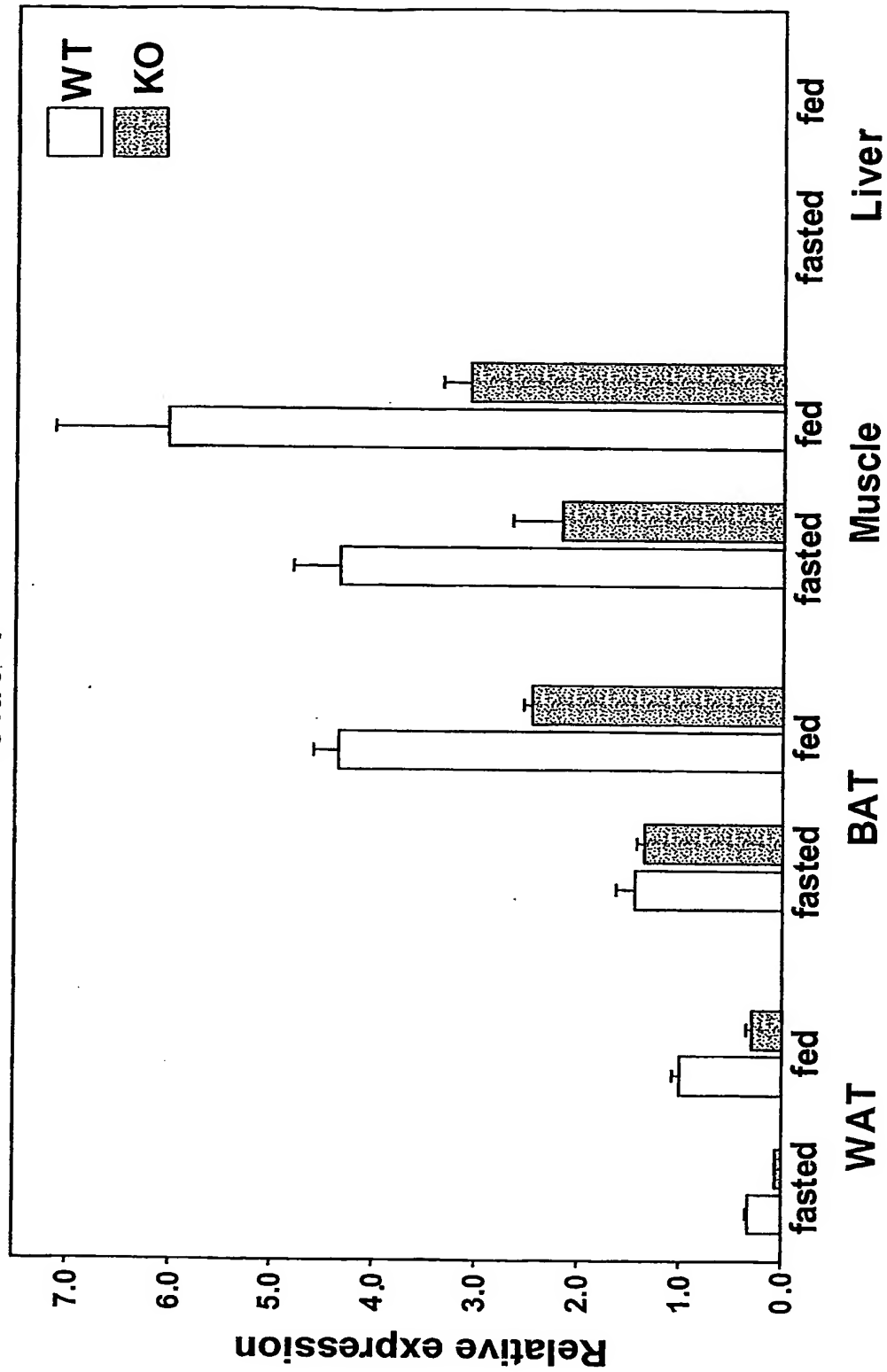
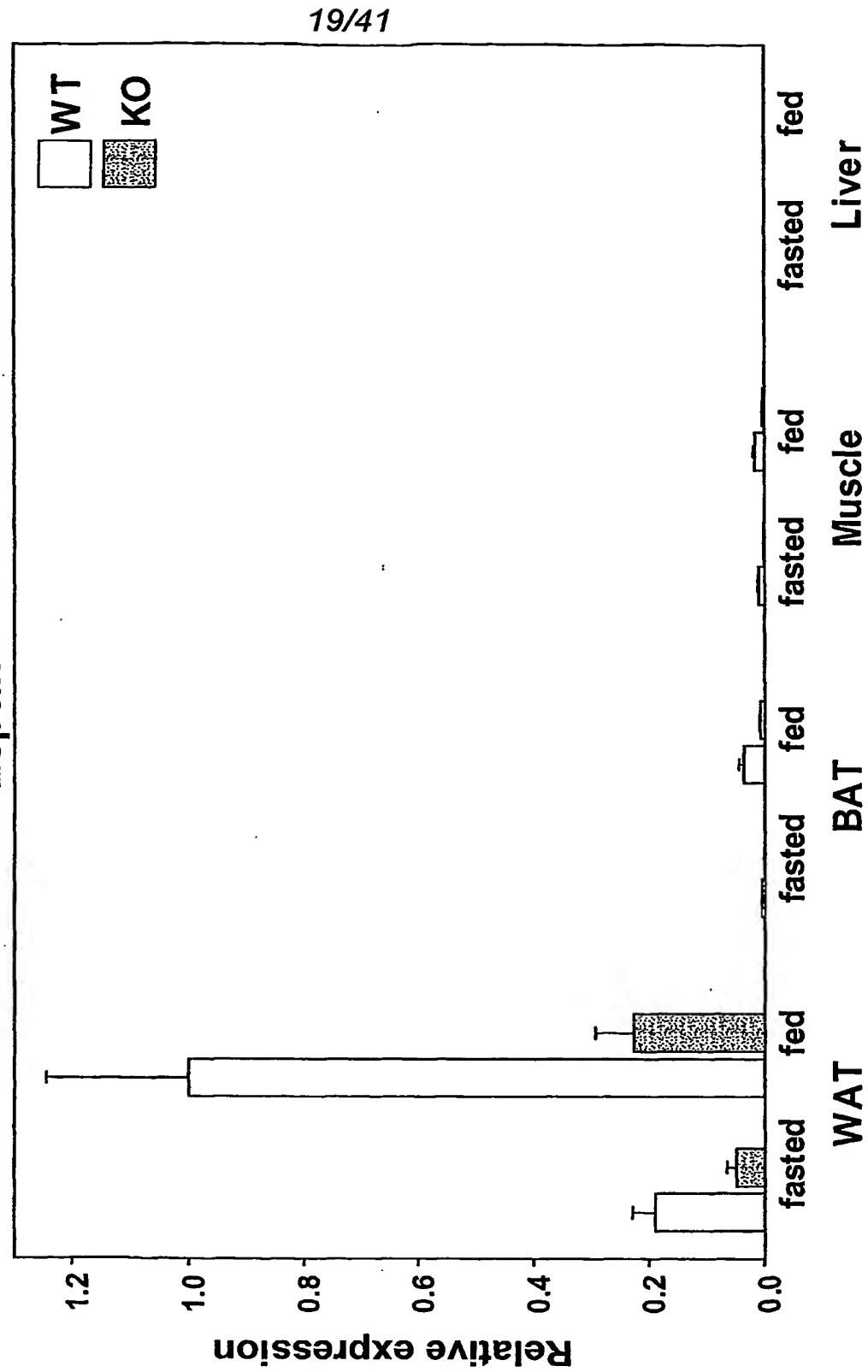
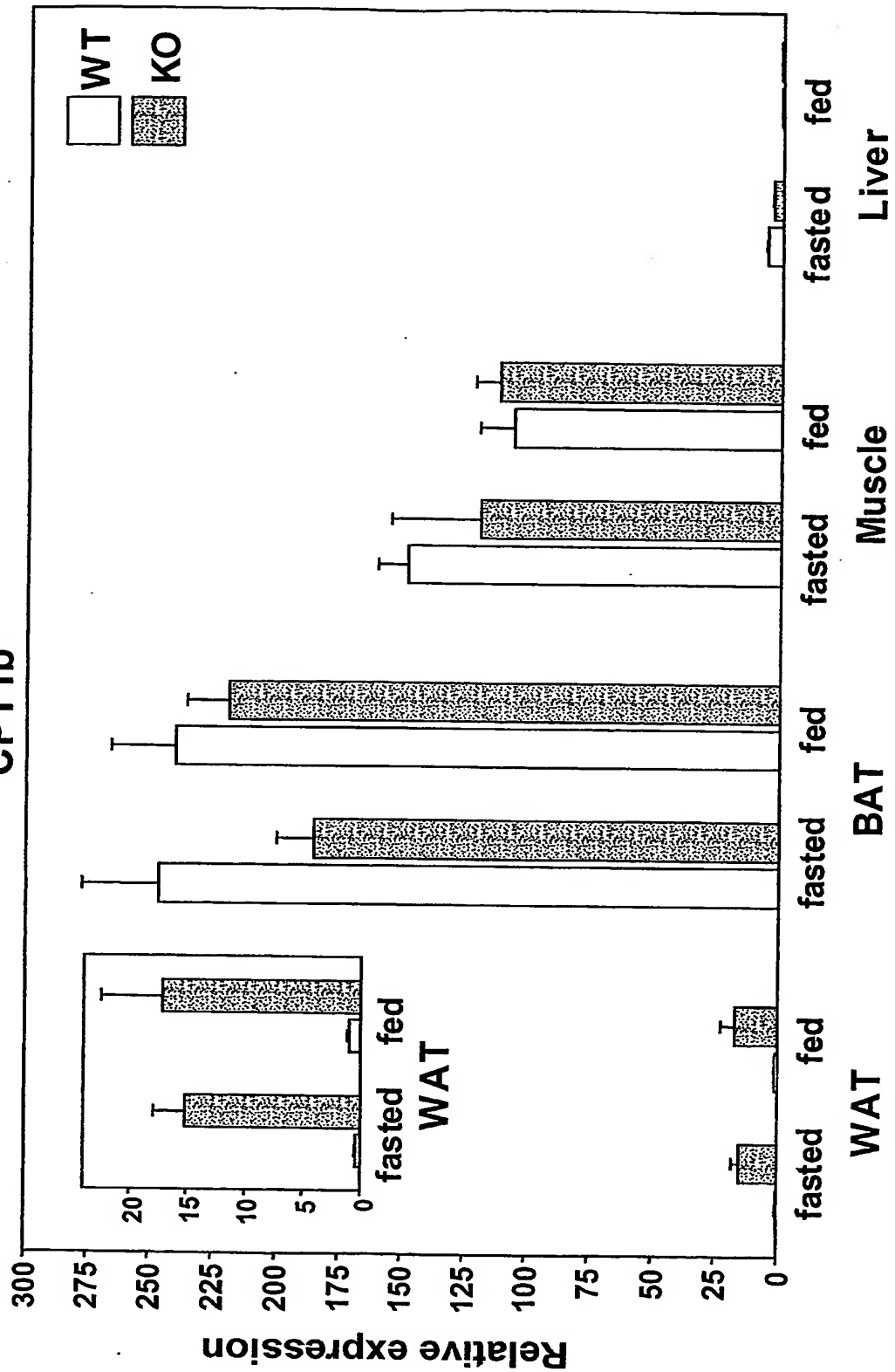


FIG. 14
Leptin



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FIG. 15
CPT1b

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FIG. 16

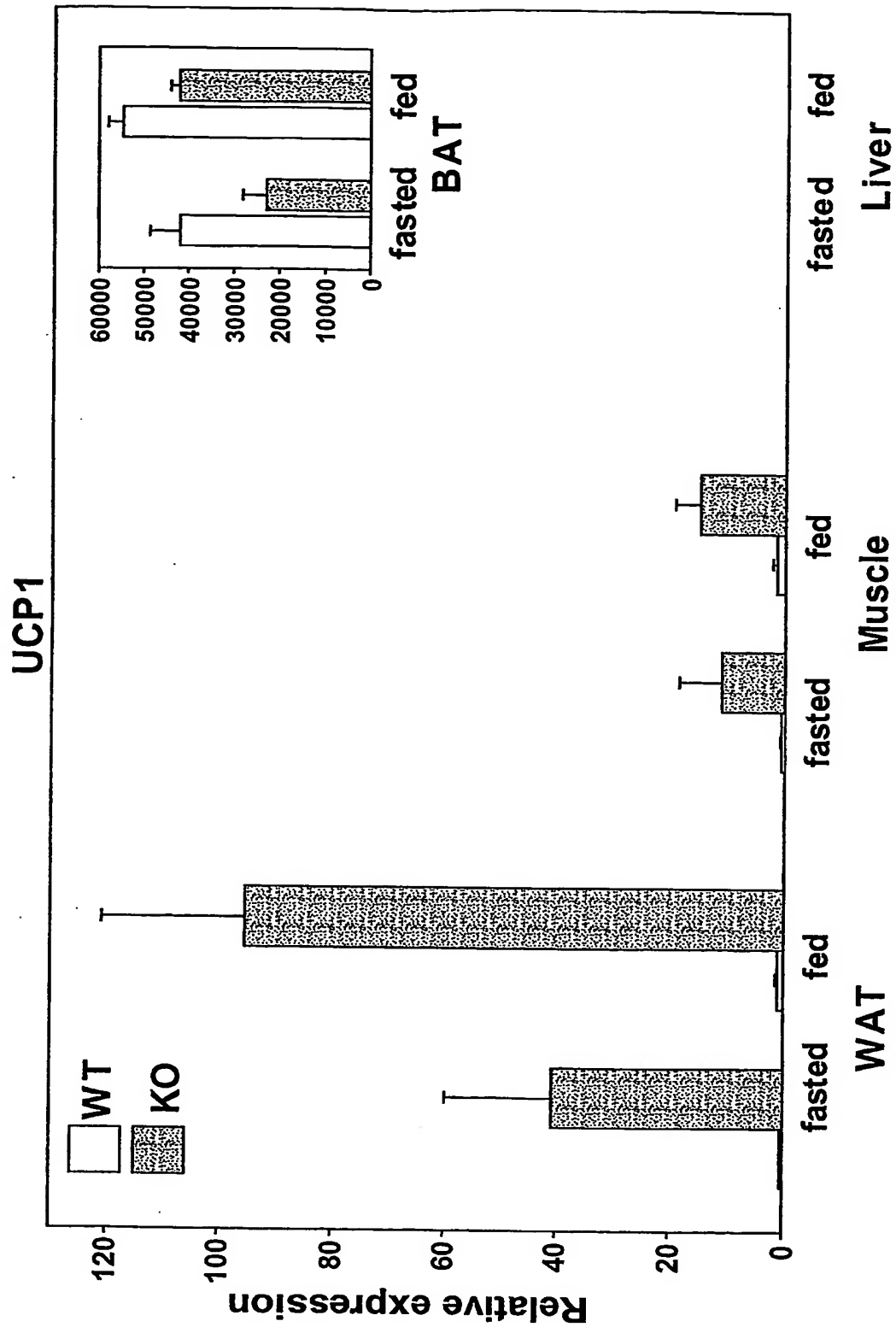
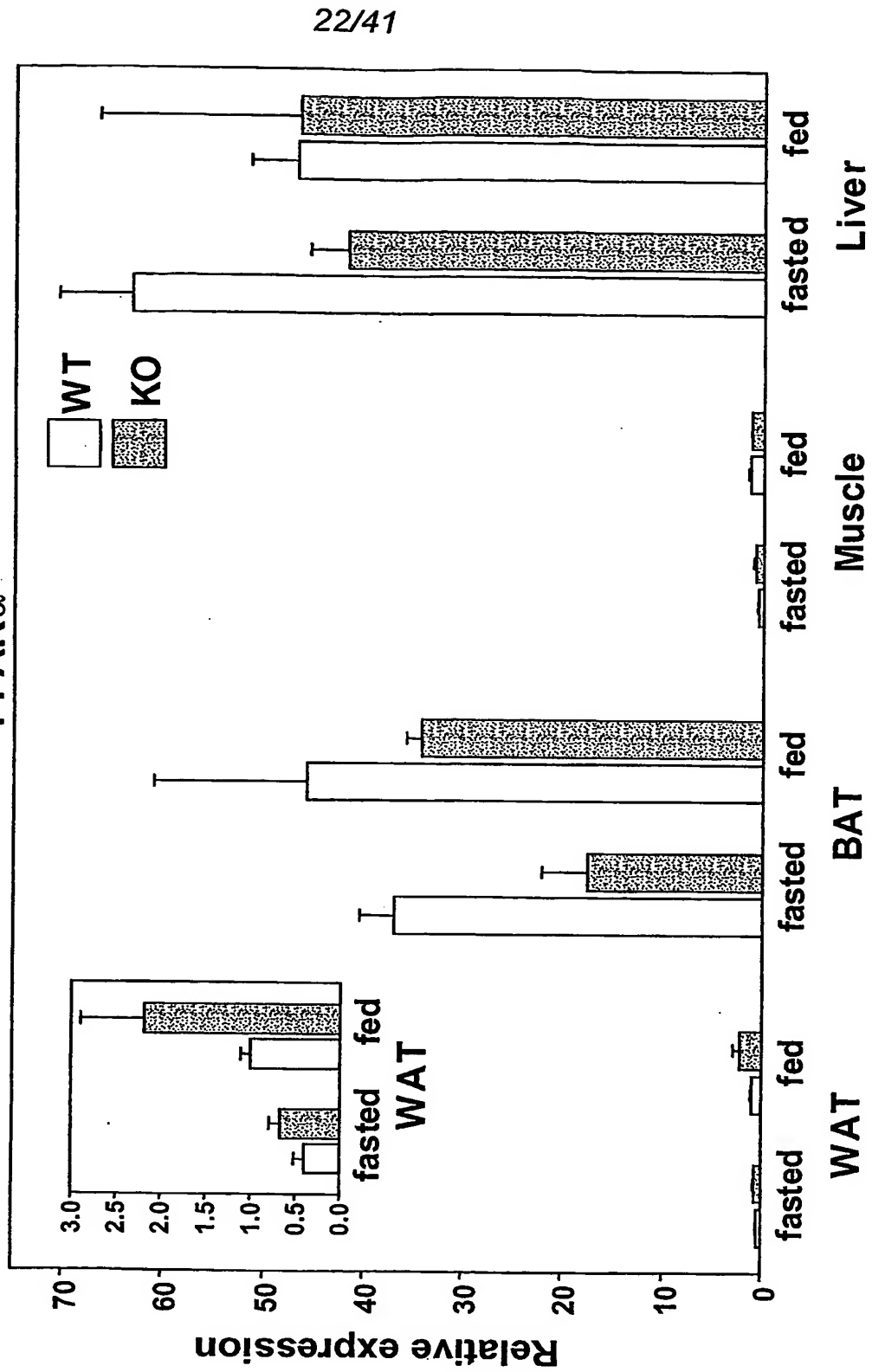


FIG. 17

PPAR α 

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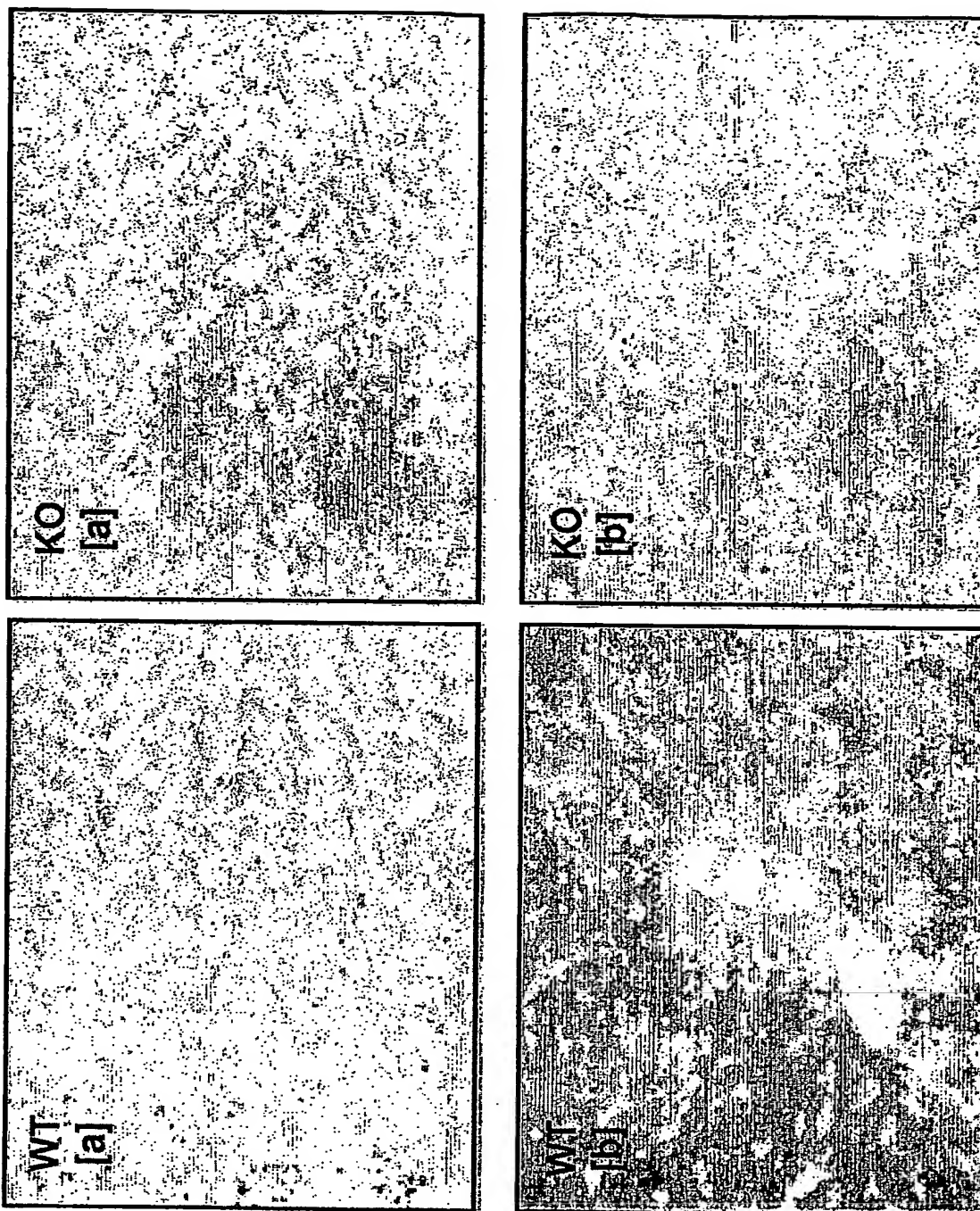


FIG. 18

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FIG. 19c

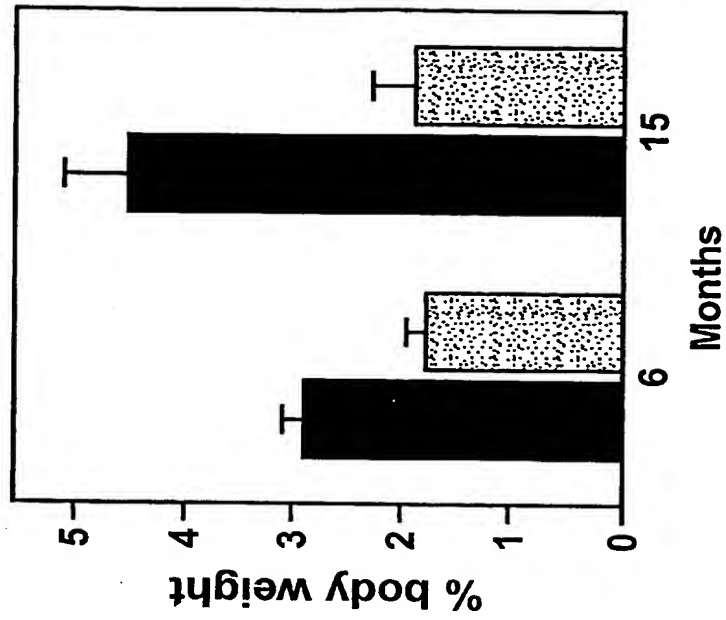
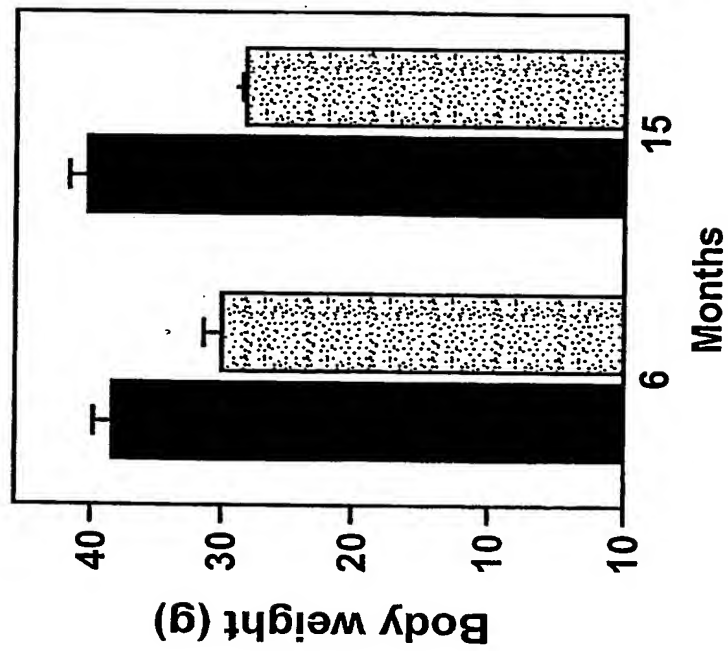


FIG. 19a



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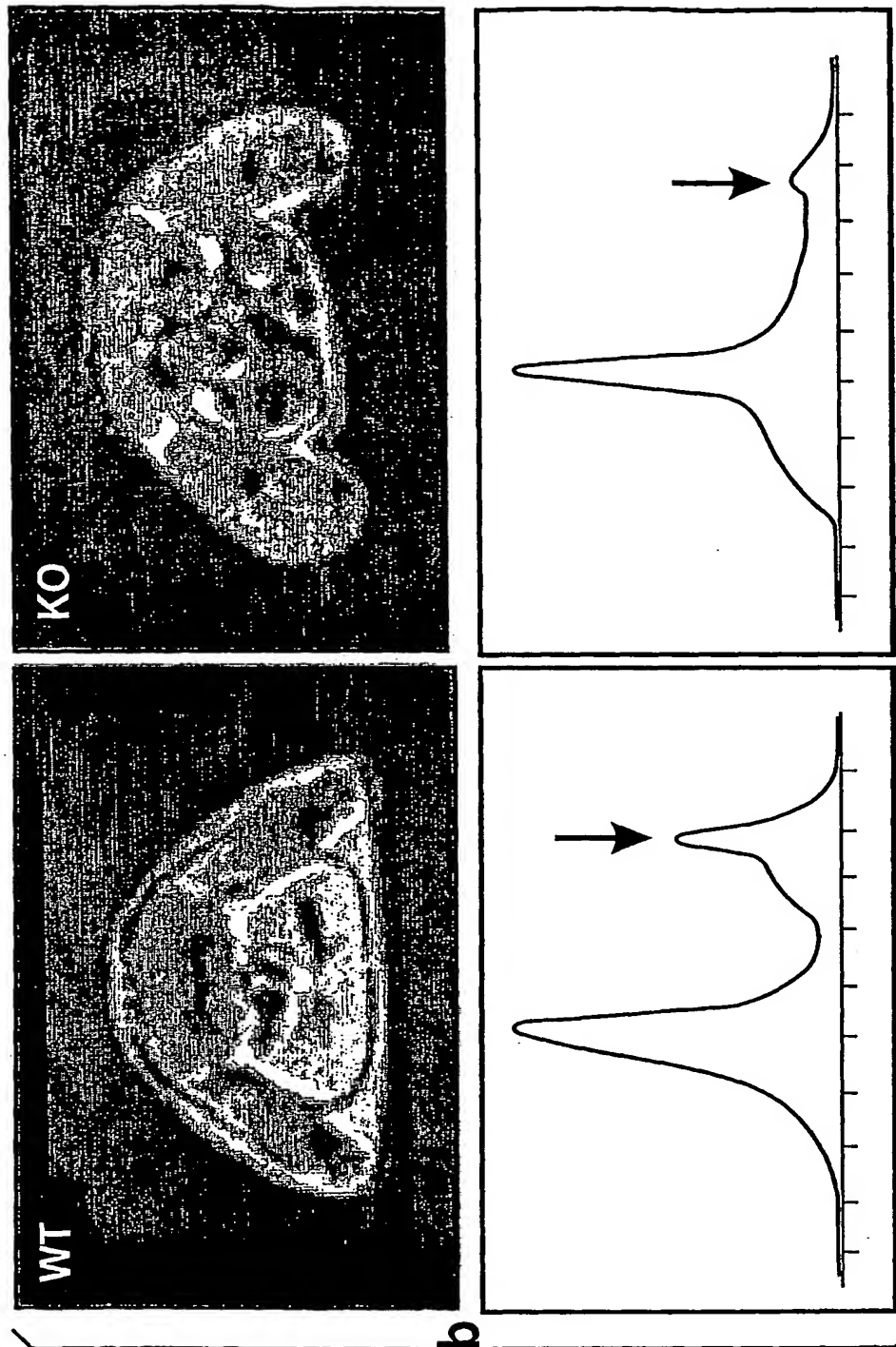


FIG. 19b

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FIG. 19d

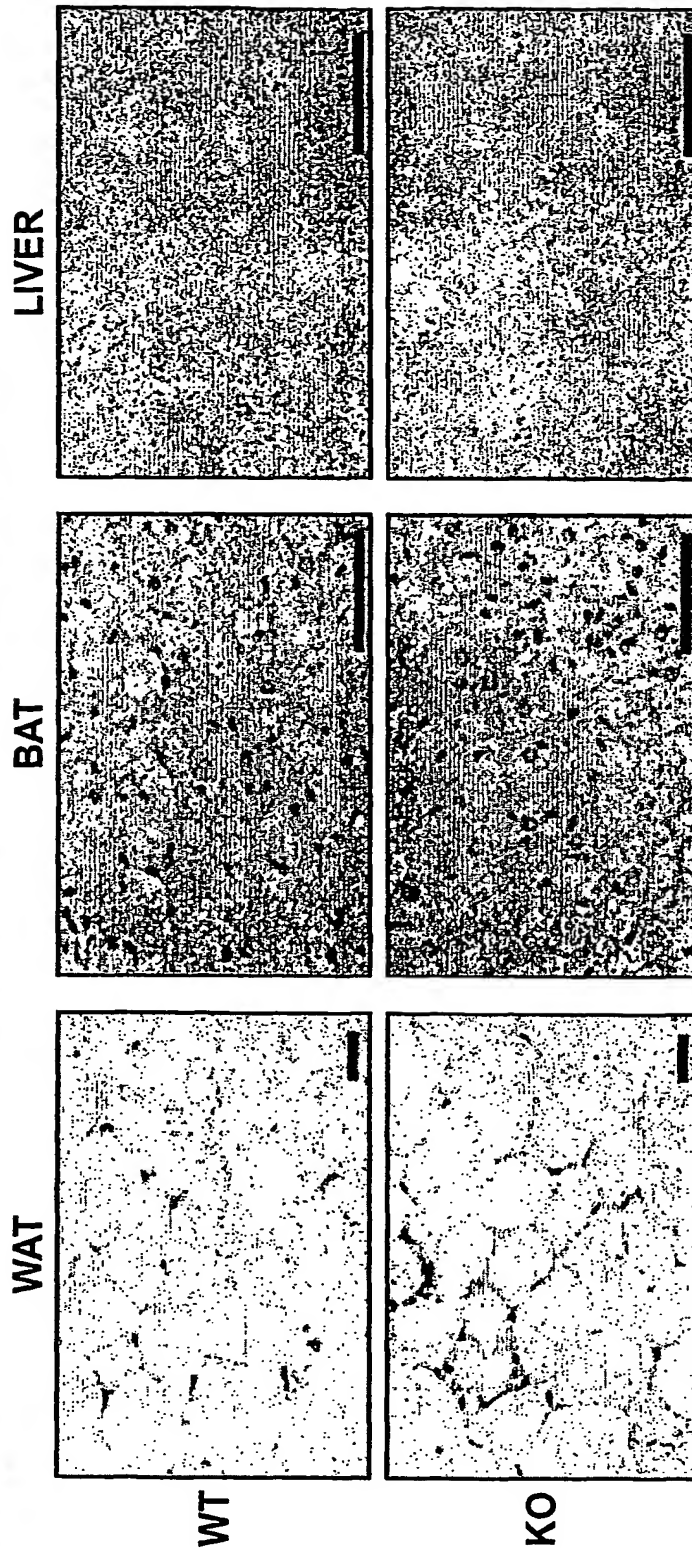


FIG. 19e

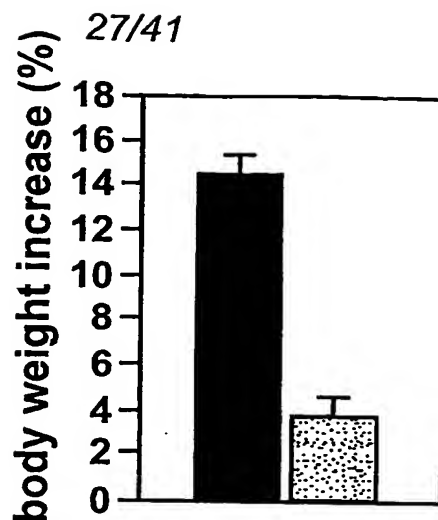


FIG. 19f

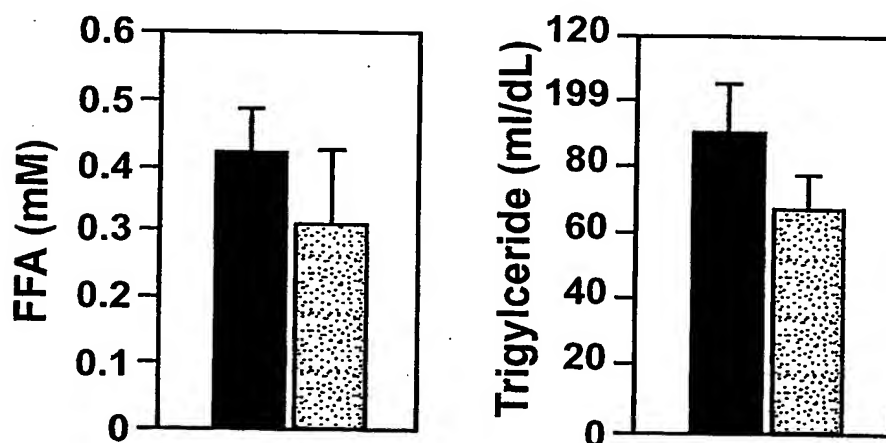
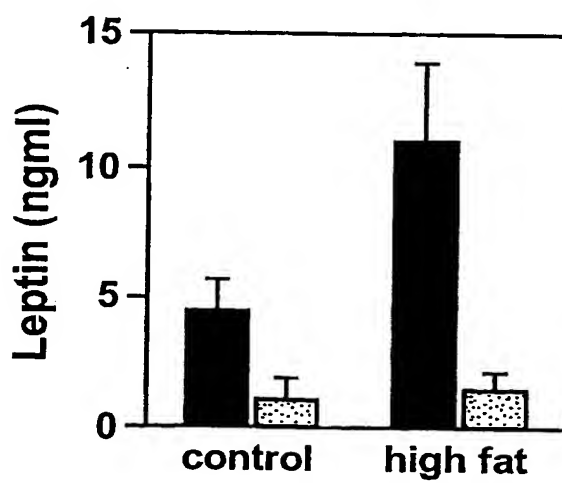


FIG. 19g



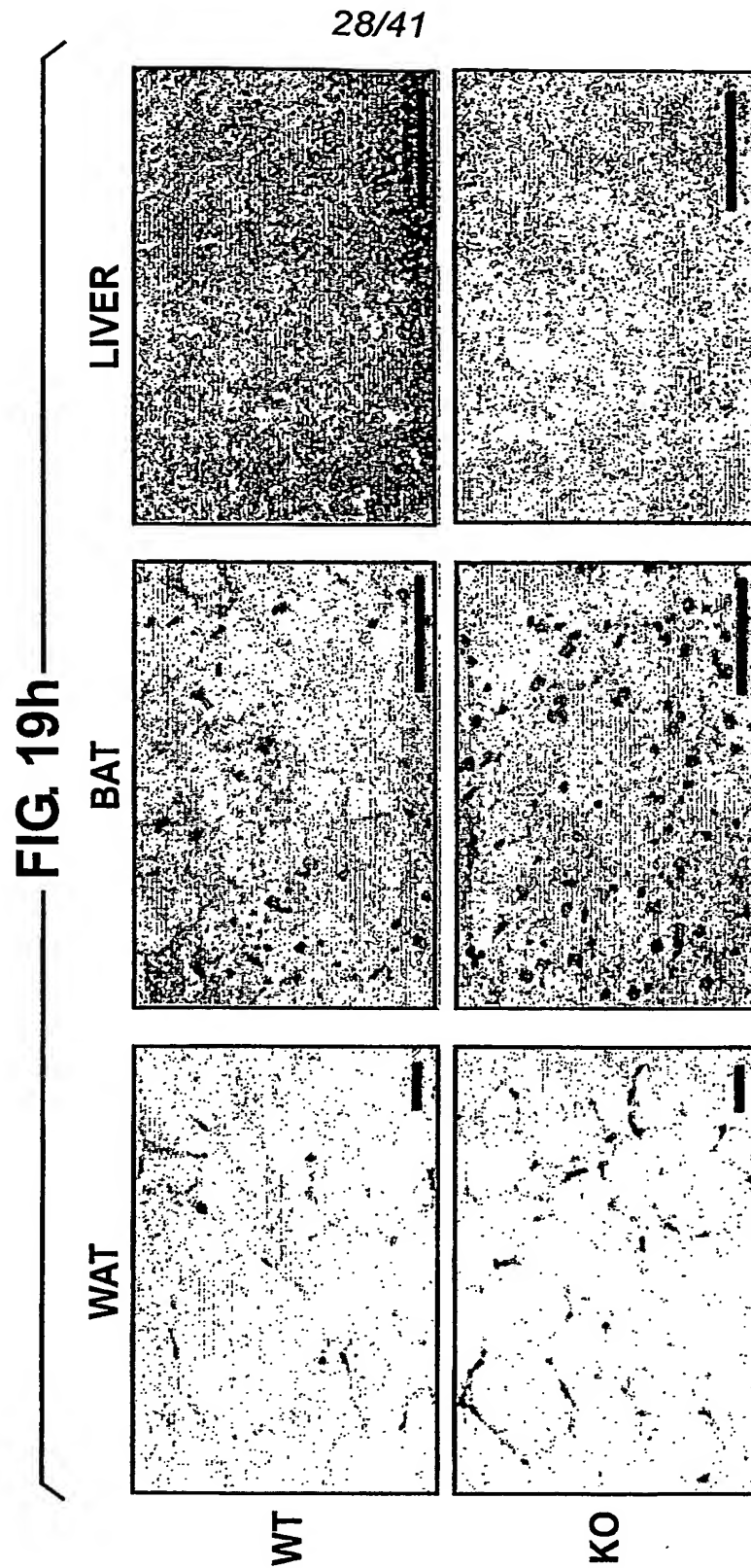
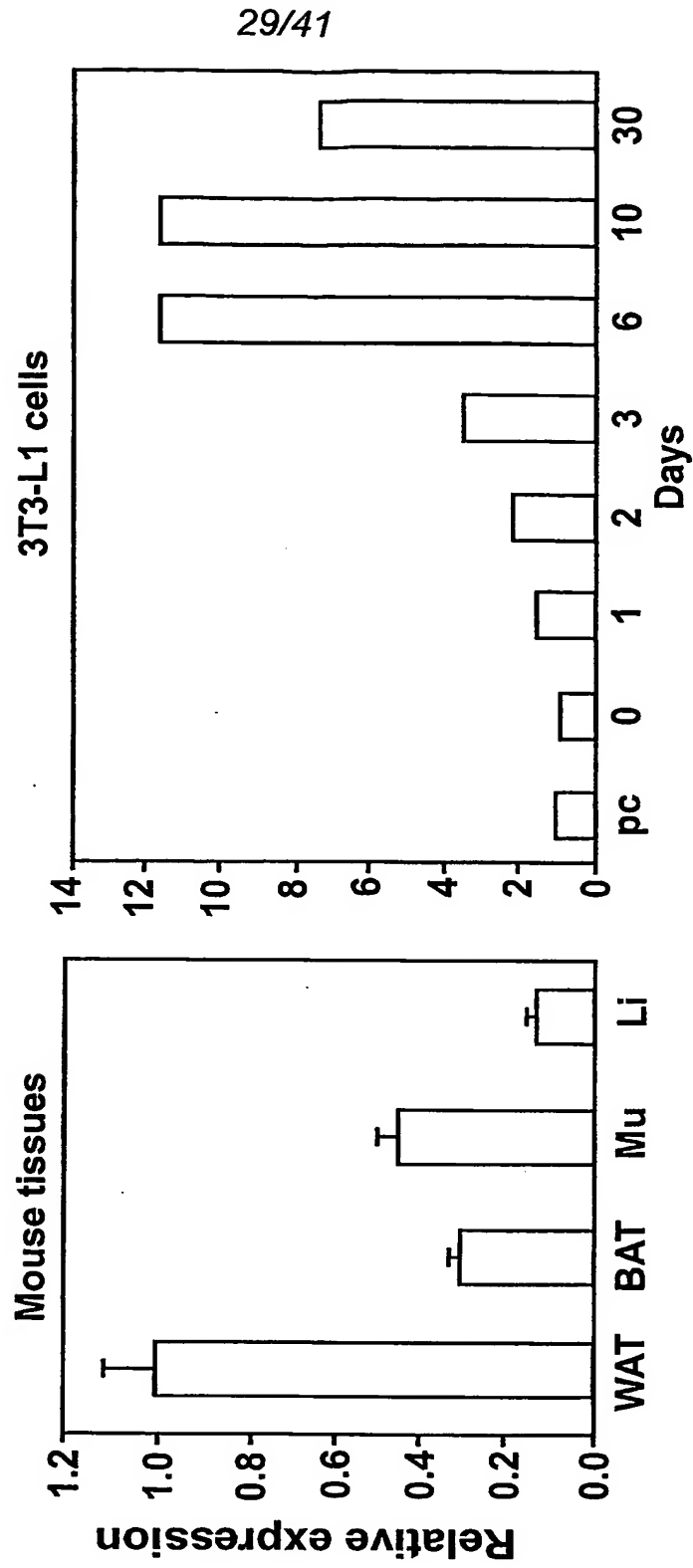
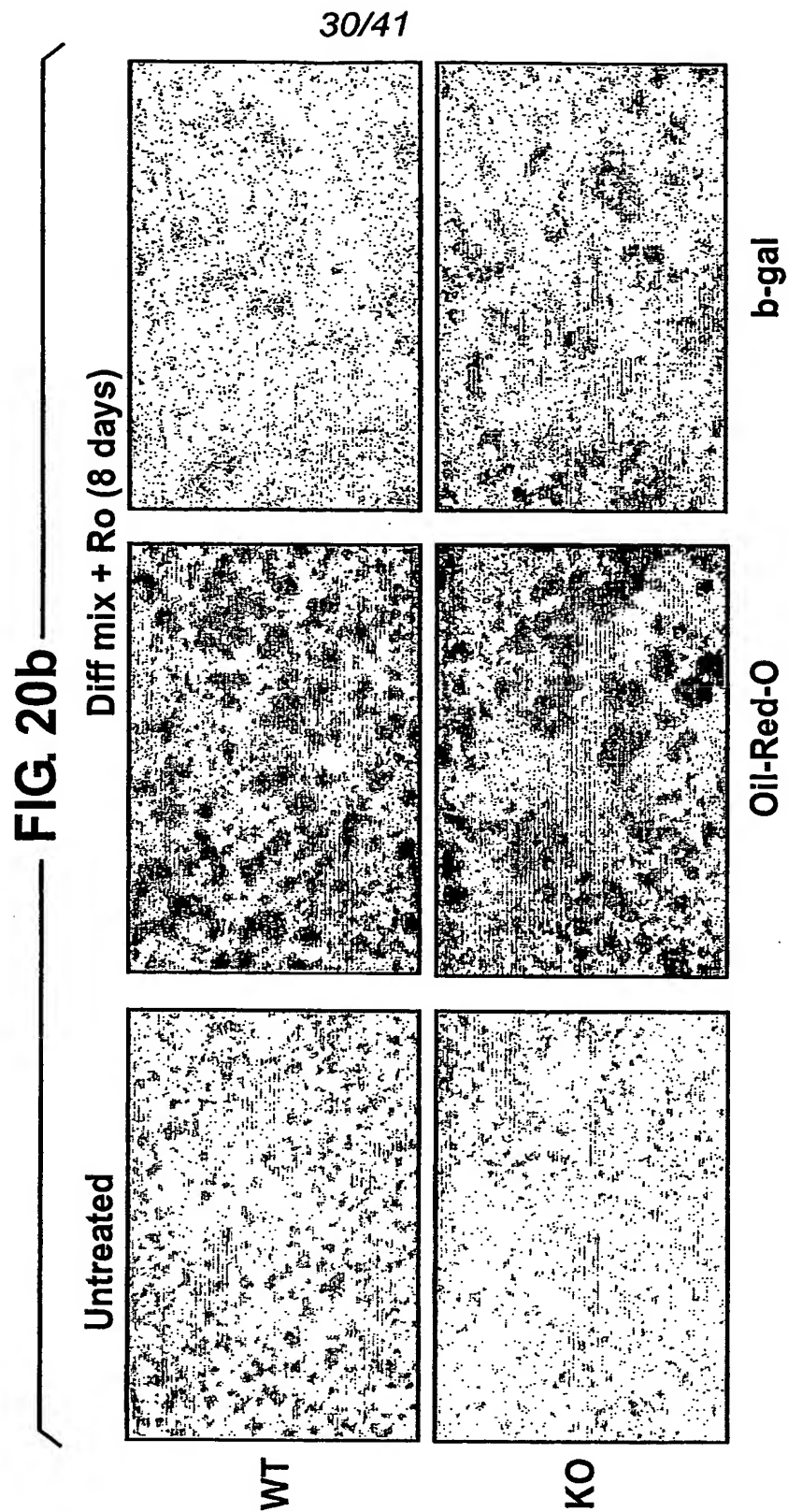
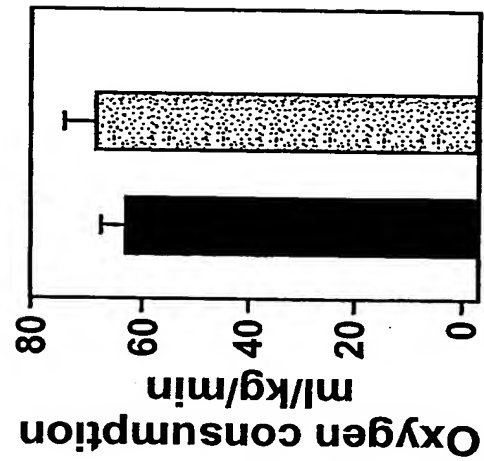
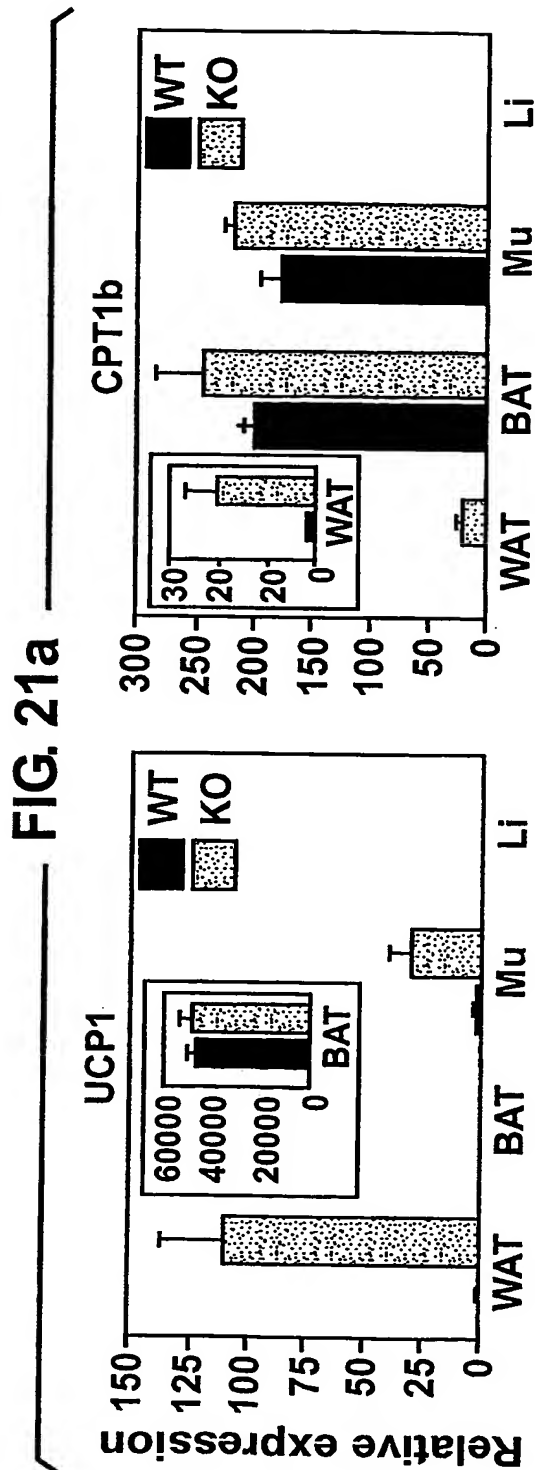


FIG. 20a



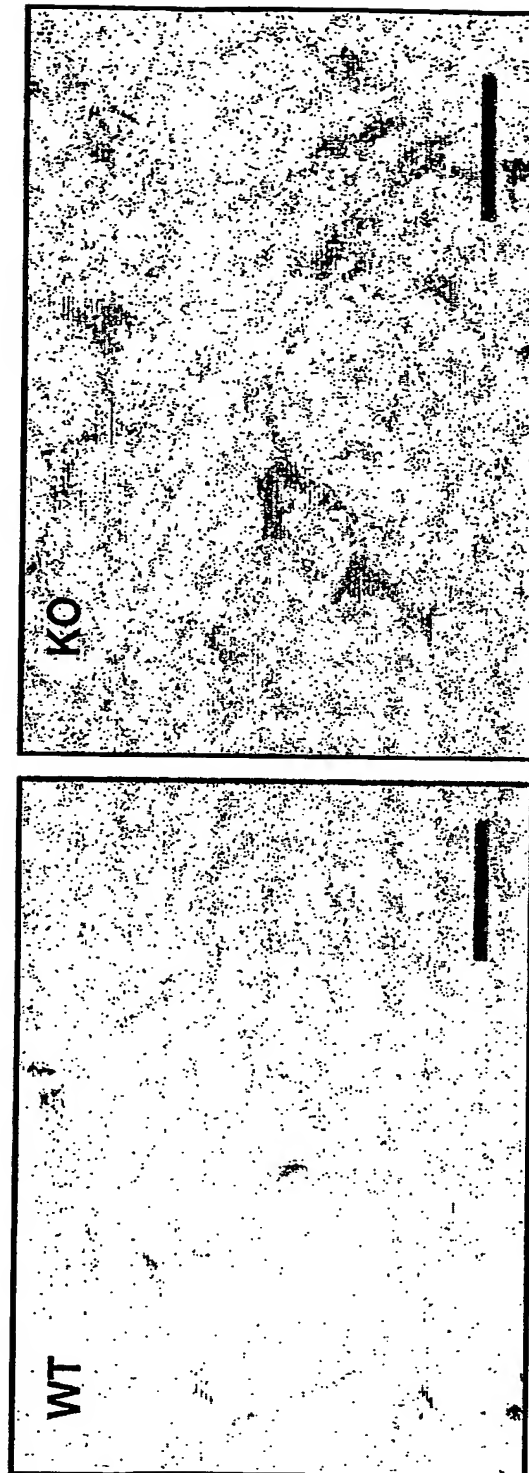


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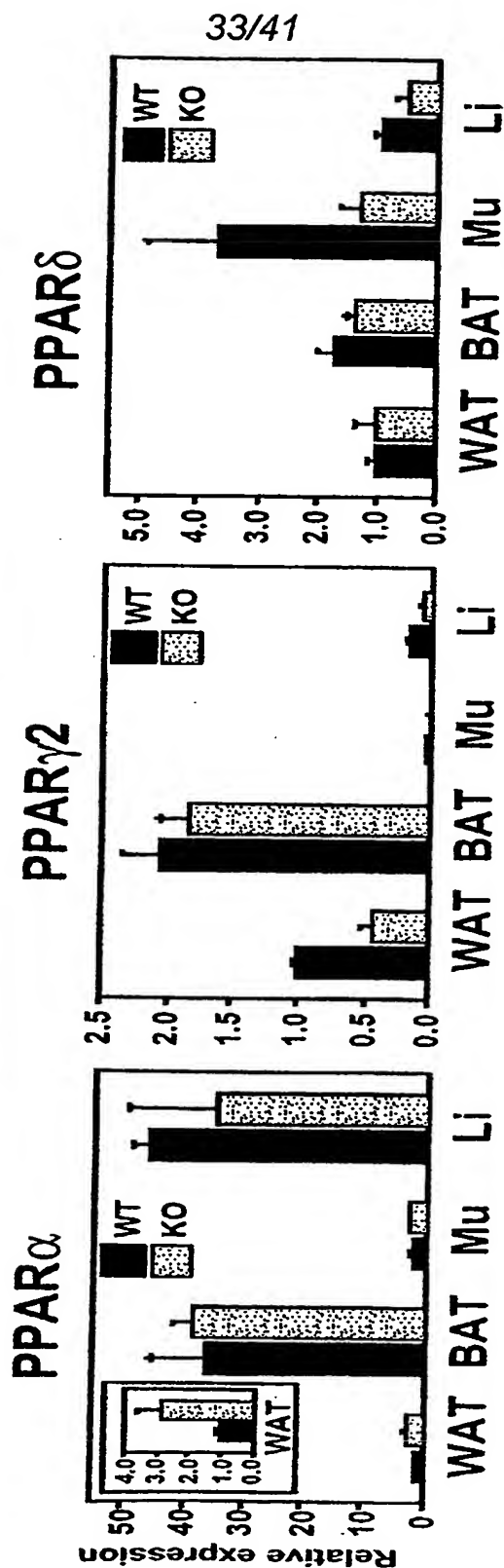
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FIG. 21b

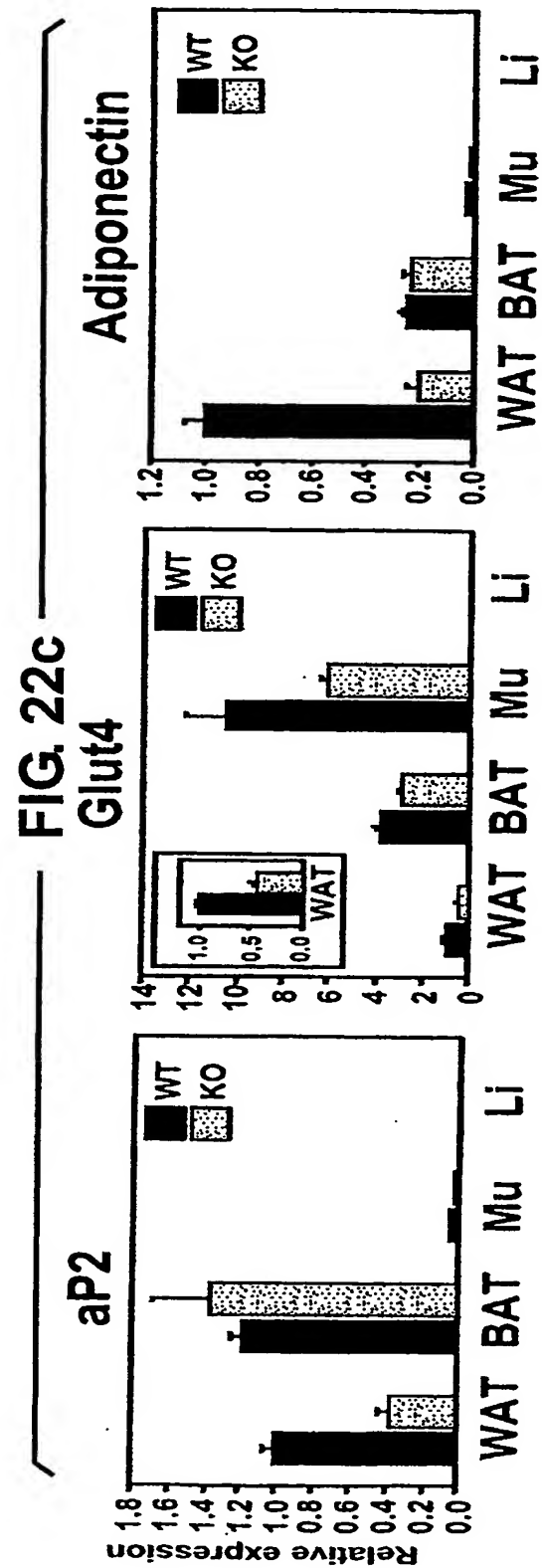
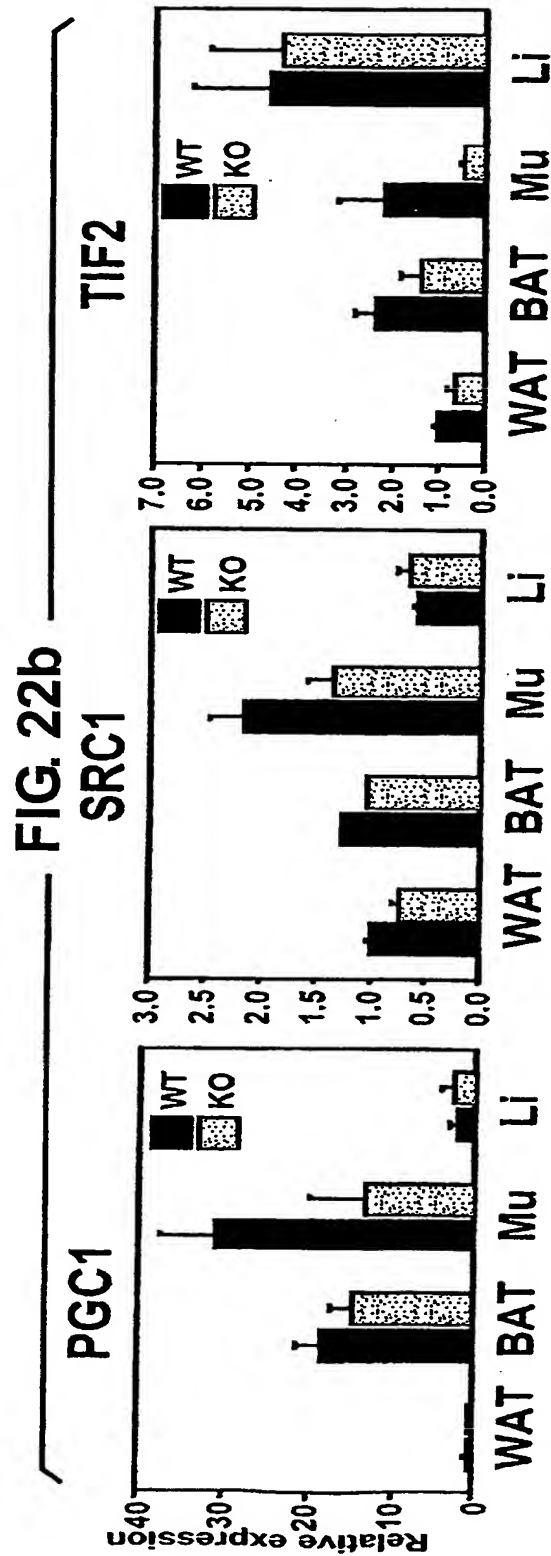


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FIG. 22a

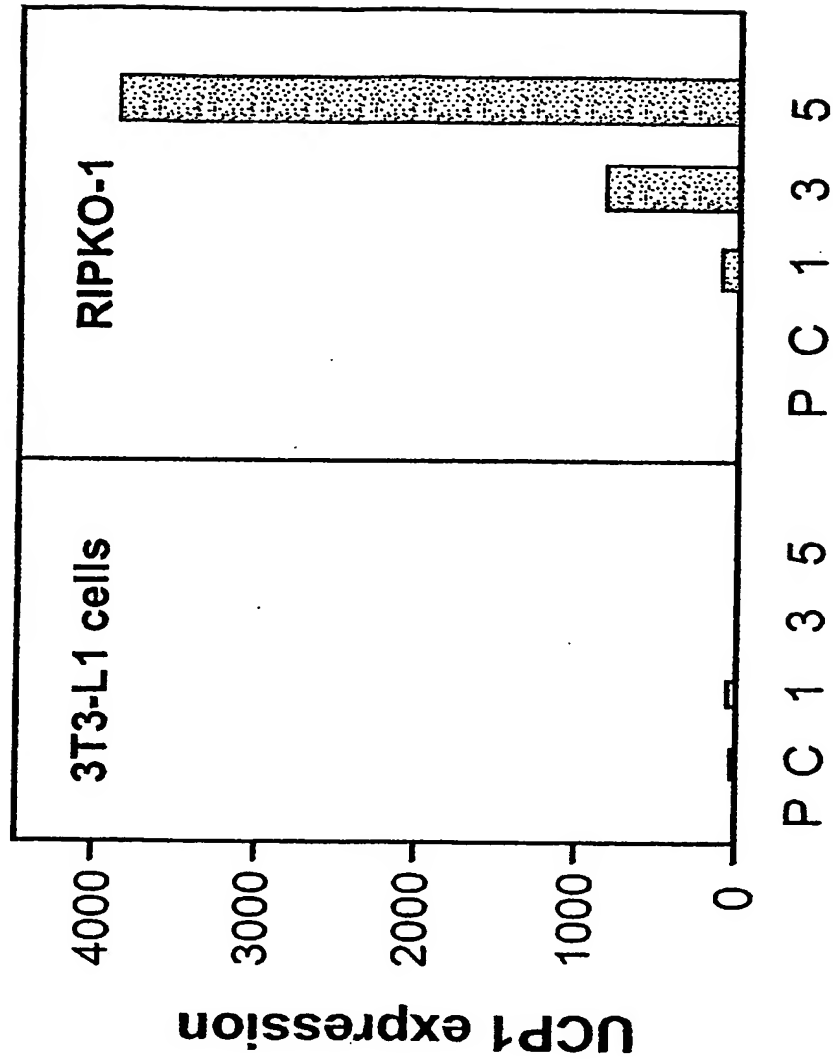


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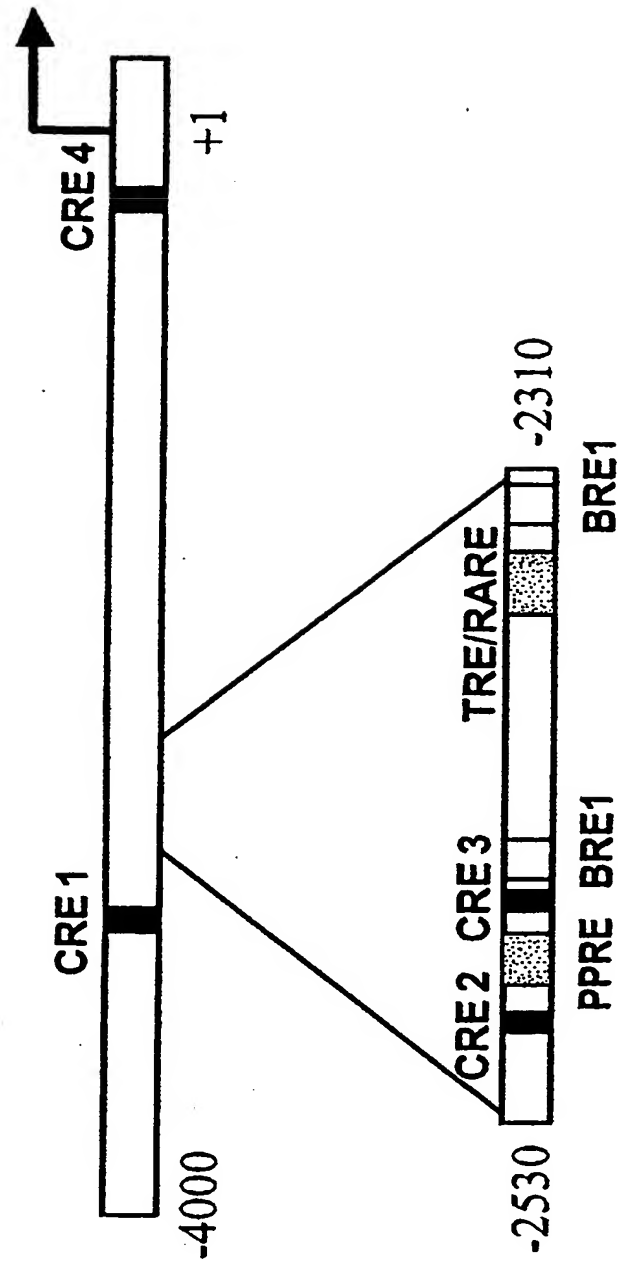
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FIG. 23a

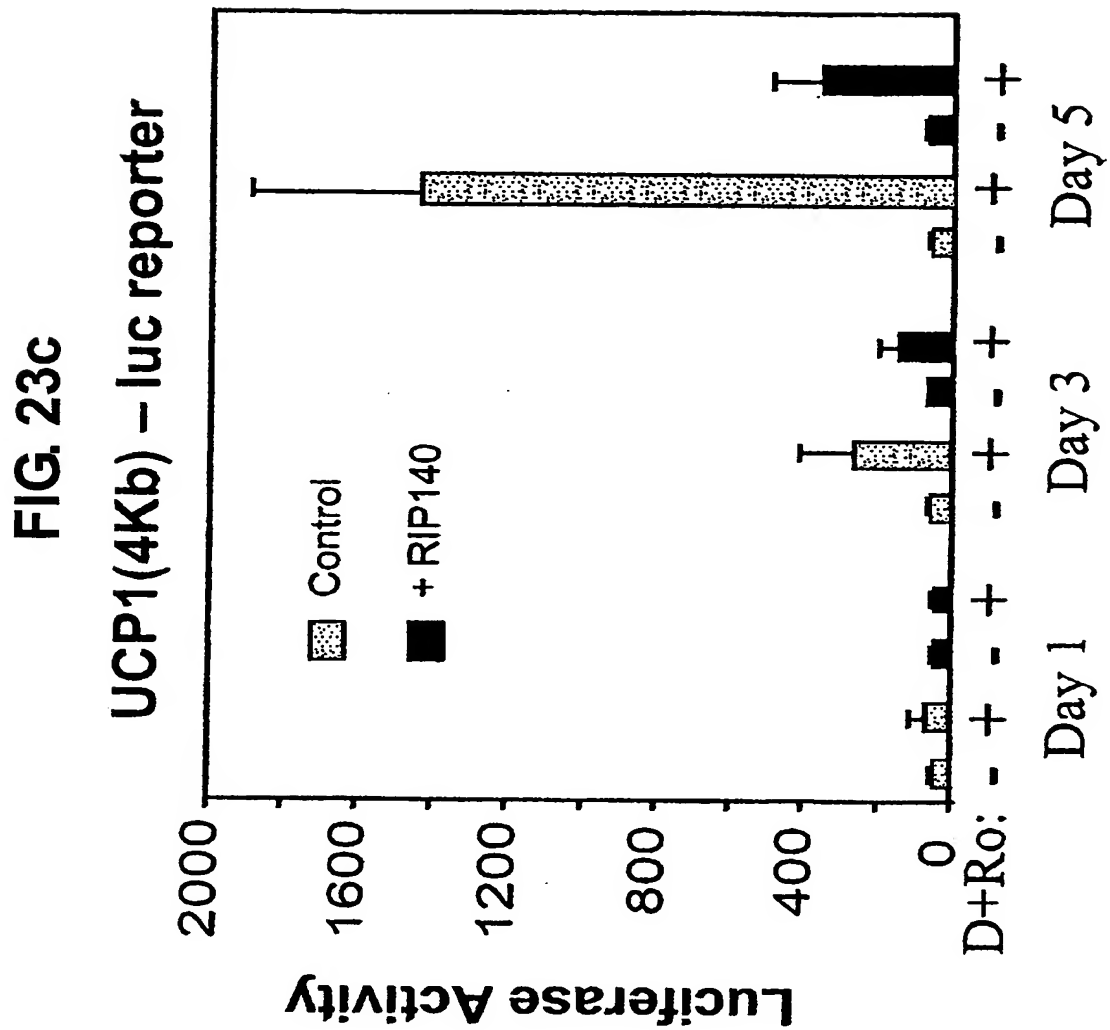


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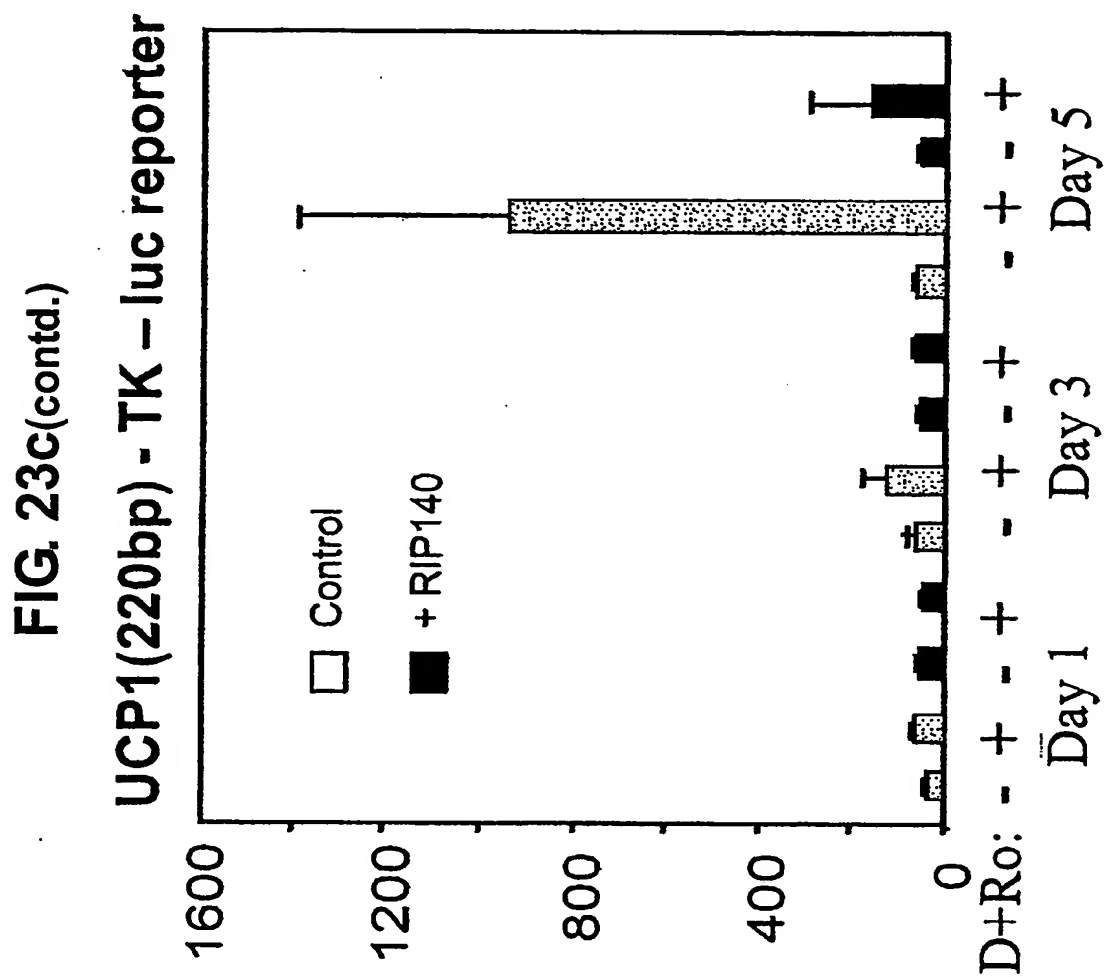
FIG. 23b



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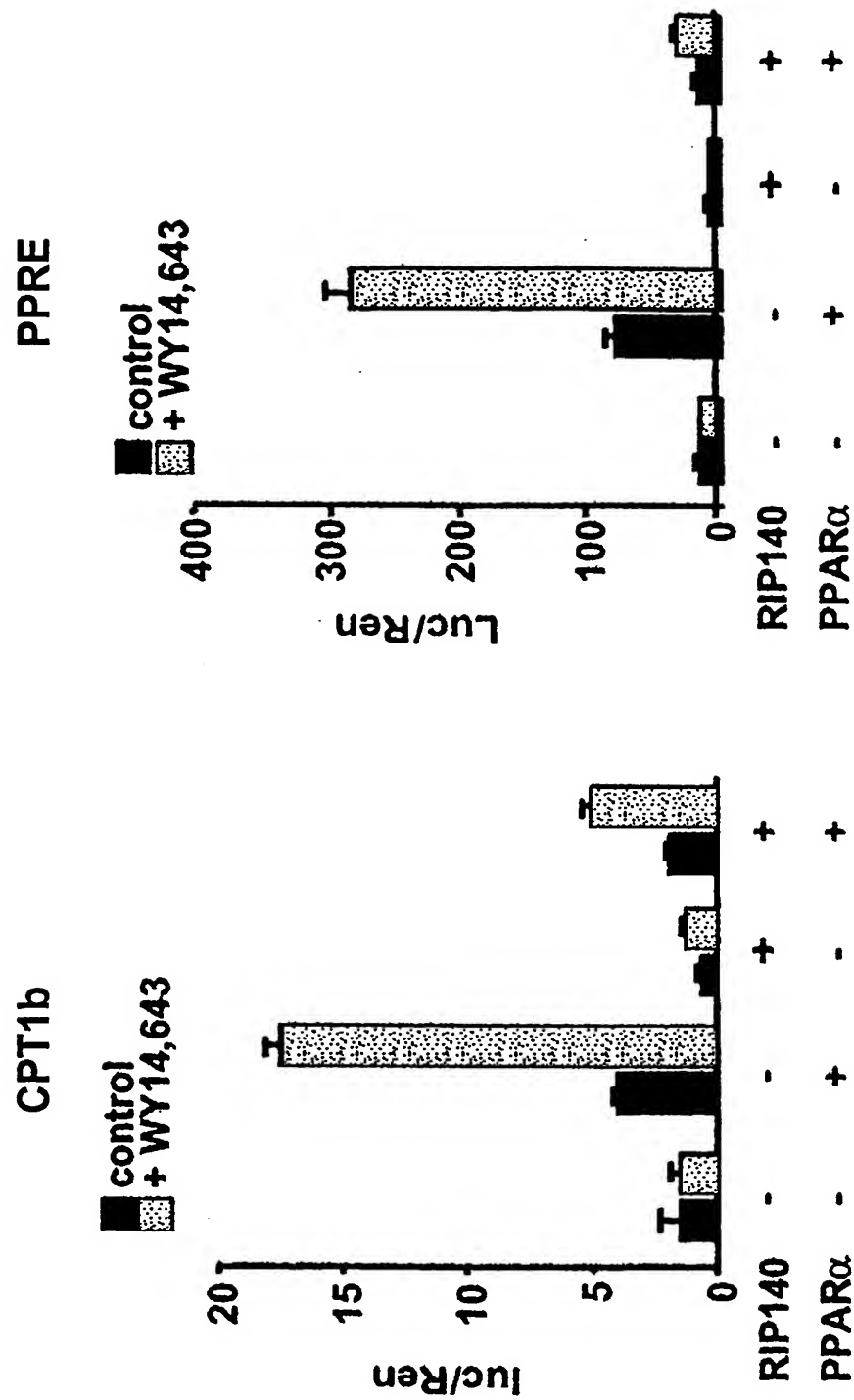


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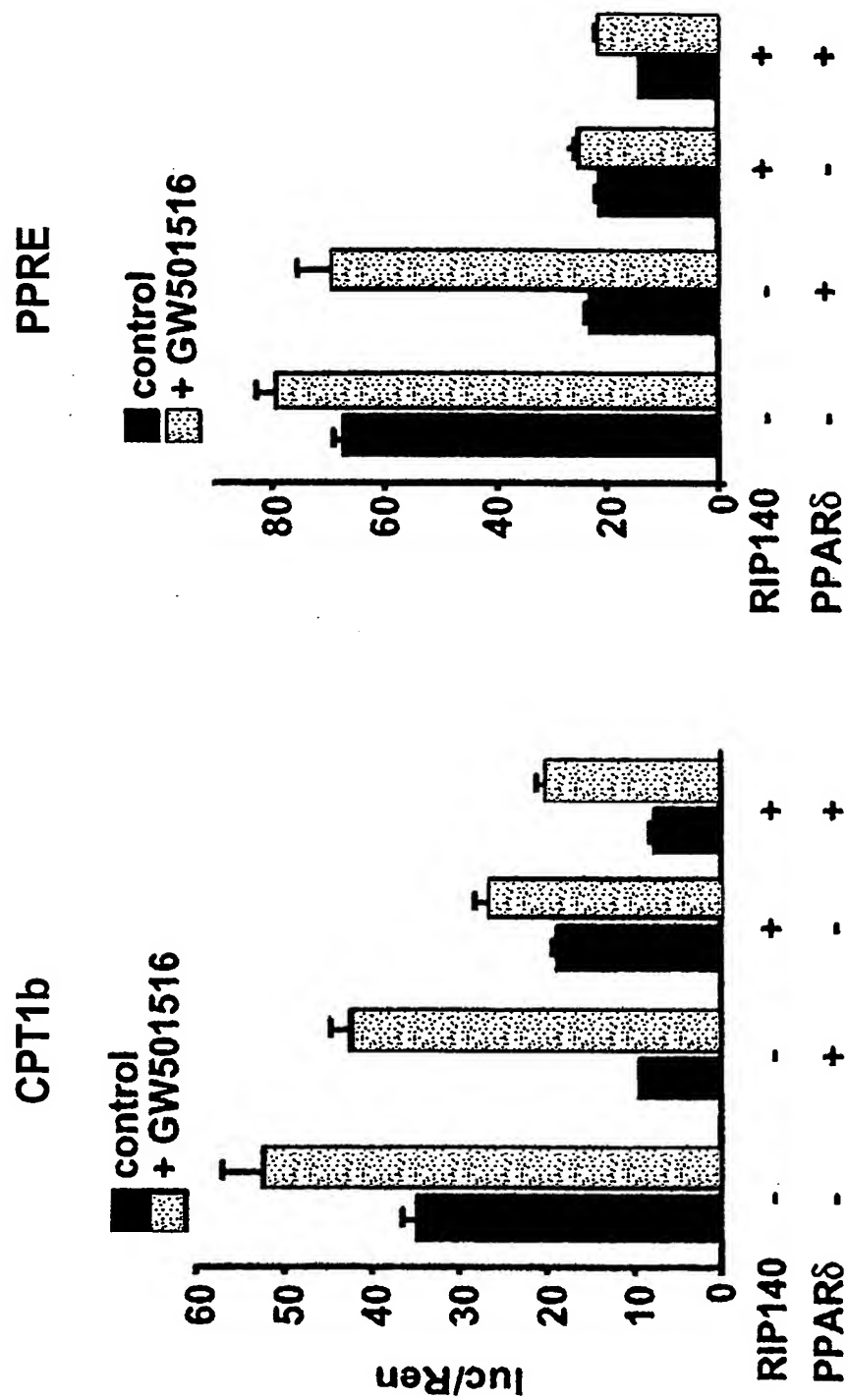
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FIG. 24a



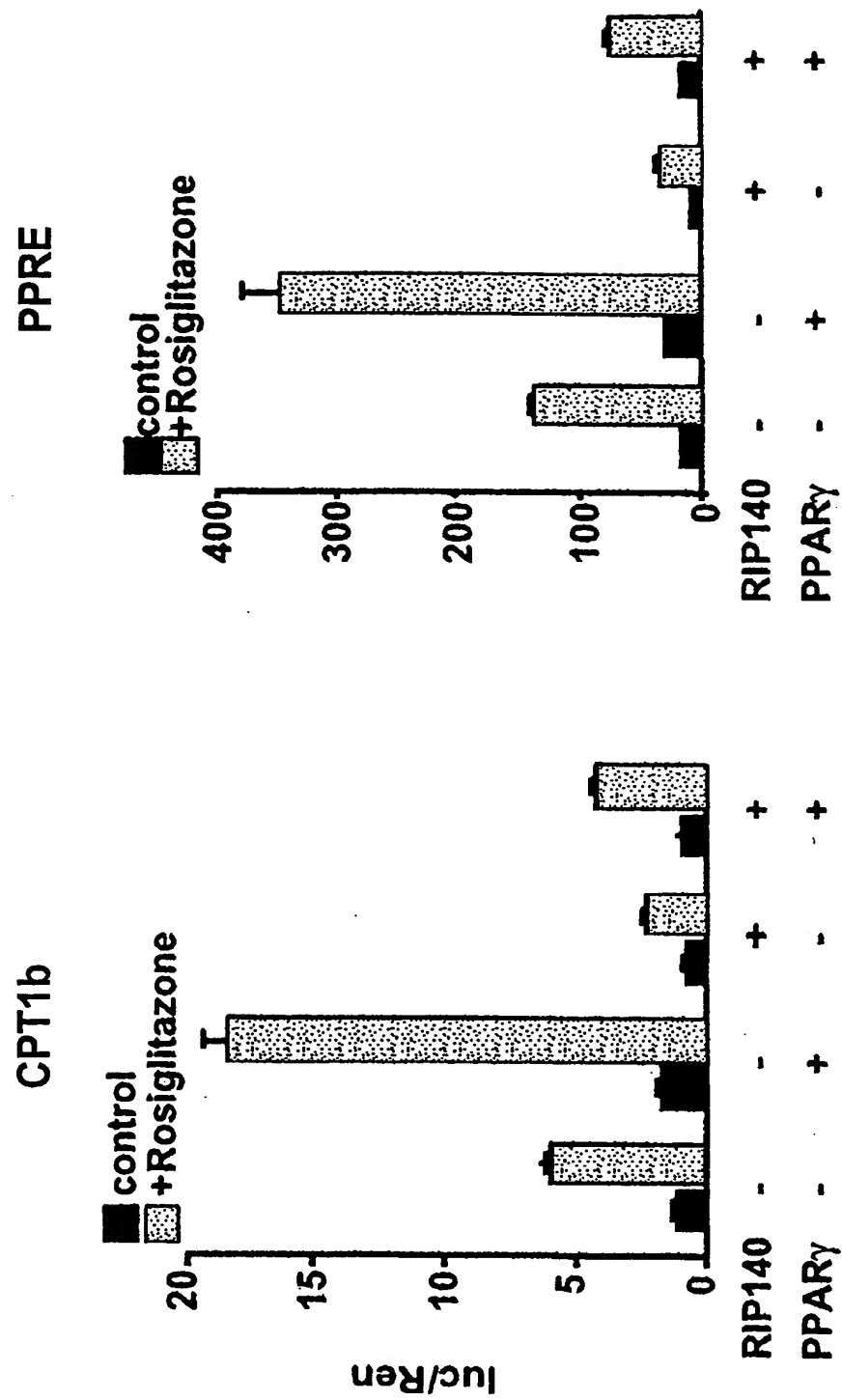
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FIG. 24b



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FIG. 24c



02 AUG 2005

INTERNATIONAL SEARCH REPORT

PCT/GB2004/000413

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N33/68 A61P3/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

EP0-Internal, BIOSIS, Sequence Search, EMBASE, PAJ, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WHITE R ET AL: "The nuclear receptor co-repressor nr1p1 (RIP140) is essential for female fertility" NATURE MEDICINE, NATURE AMERICA, NEW YORK, US, vol. 6, no. 12, December 2000 (2000-12), pages 1368-1374, XP002258567 ISSN: 1078-8956 abstract page 1369, column 1; figure 2	1-11,15, 16
A	EP 1 267 171 A (INST NAT SANTE RECH MED) 18 December 2002 (2002-12-18) paragraphs [0009], [0031], [0163]; figures 3,4; example 6 ----- -/--	1-11,15, 16

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "8" document member of the same patent family

Date of the actual completion of the international search

13 May 2004

Date of mailing of the international search report

14 OCT 2004

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INTERNATIONAL SEARCH REPORT

PCT/GB2004/000413

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2002/004205 A1 (GRAY JOHN G ET AL) 10 January 2002 (2002-01-10) paragraphs [0018] - [0021]; figures 2-4,6 -----	1-11,15, 16
A	US 6 266 622 B1 (APRILETTI JAMES J ET AL) 24 July 2001 (2001-07-24) example 3; table 3 -----	1-11,15, 16
A	YAMAUCHI TOSHIMASA ET AL: "The mechanisms by which both heterozygous peroxisome proliferator-activated receptor gamma (PPARgamma) deficiency and PPARgamma agonist improve insulin resistance" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 276, no. 44, 2 November 2001 (2001-11-02), pages 41245-41254, XP002279645 ISSN: 0021-9258 abstract page 41245, column 2 page 41253, column 2, paragraph 4 -----	1
A	PALOU A ET AL: "Obesity: Molecular bases of a multifactorial problem" EUROPEAN JOURNAL OF NUTRITION, vol. 39, no. 4, August 2000 (2000-08), pages 127-144, XP002279646 ISSN: 1436-6207 page 135 - page 136, column 1; table 1 -----	1

INTERNATIONAL SEARCH REPORT

PCT/GB2004/000413

Box II Observations where certain claims were found unsearchable (Continuation of Item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 17,22
because they relate to subject matter not required to be searched by this Authority, namely:
Although claims 1-11 directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the binding between RIP140 and modulation of fat storage.
2. ☒ Claims Nos.: 12-14,17-22
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of Item 3 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-12 , 13, 14-22 partially

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box II.1

Although claims 1-11 directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the binding between RIP140 and modulation of fat storage.

Continuation of Box II.1

Claims Nos.: 17,22

Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy

Continuation of Box II.2

Claims Nos.: 12-14,17-22

Present claims 12-14, 17-22 and 15-16 (in as far as they depend on claims 12-14) relate to a compound/pharmaceutical composition and their use defined by reference to a desirable characteristic or property, namely obtained or obtainable by a method of claim 1.

The claims cover all compounds/pharmaceutical compositions and their use having this characteristic or property, whereas the application provides no support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for such compounds/pharmaceutical compositions. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the compound/pharmaceutical composition by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, no search has been carried out for claims 12-14 and 17-22 and claims 15-16, in as far as they depend on claims 12-14.

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure. If the application proceeds into the regional phase

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

before the EPO, the applicant is reminded that a search may be carried out during examination before the EPO (see EPO Guideline C-VI, 8.5), should the problems which led to the Article 17(2) declaration be overcome.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: Claims 1-12 (partially), 13,14-22 (partially)

Screening for compounds that modulate fat storage in a mammal comprising identifying a compound which binds to RIP140, compounds and pharmaceutical compositions derived therefrom as well as their use.

2. claims: Claims 1-12 (partially), 14-22 (partially)

Screening for compounds that modulate fat storage in a mammal comprising identifying a compound which binds to the RIP140 target protein AhR, compounds and pharmaceutical compositions derived therefrom as well as their use.

3. claims: Claims 1-12 (partially), 14-22 (partially)

Screening for compounds that modulate fat storage in a mammal comprising identifying a compound which binds to the RIP140 target protein ER, compounds and pharmaceutical compositions derived therefrom as well as their use.

4. claims: Claims 1-12 (partially), 14-22 (partially)

Screening for compounds that modulate fat storage in a mammal comprising identifying a compound which binds to the RIP140 target protein RAR, compounds and pharmaceutical compositions derived therefrom as well as their use.

5. claims: Claims 1-12 (partially), 14-22 (partially)

Screening for compounds that modulate fat storage in a mammal comprising identifying a compound which binds to the RIP140 target protein TR, compounds and pharmaceutical compositions derived therefrom as well as their use.

6. claims: Claims 1-12 (partially), 14-22 (partially)

Screening for compounds that modulate fat storage in a mammal comprising identifying a compound which binds to the RIP140 target protein RXR, compounds and pharmaceutical compositions derived therefrom as well as their use.

7. claims: Claims 1-12 (partially), 14-22 (partially)

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Screening for compounds that modulate fat storage in a mammal comprising identifying a compound which binds to the RIP140 target protein VDR, compounds and pharmaceutical compositions derived therefrom as well as their use.

8. claims: Claims 1-12 (partially), 14-22 (partially)

Screening for compounds that modulate fat storage in a mammal comprising identifying a compound which binds to the RIP140 target protein PPAR, compounds and pharmaceutical compositions derived therefrom as well as their use.

9. claims: Claims 1-12 (partially), 14-22 (partially)

Screening for compounds that modulate fat storage in a mammal comprising identifying a compound which binds to the RIP140 target protein SF-1, compounds and pharmaceutical compositions derived therefrom as well as their use.

10. claims: Claims 1-12 (partially), 14-22 (partially)

Screening for compounds that modulate fat storage in a mammal comprising identifying a compound which binds to the RIP140 target protein DAX-1, compounds and pharmaceutical compositions derived therefrom as well as their use.

INTERNATIONAL SEARCH REPORT

PCT/GB2004/000413

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